PURIFIED AND ISOLATED POTASSIUM-CHLORIDE COTRANSPORTER NUCLEIC ACIDS AND POLYPEPTIDES AND THERAPEUTIC AND SCREENING METHODS USING SAME

APPLICATION FOR UNITED STATES
LETTERS PATENT

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Description

PURIFIED AND ISOLATED POTASSIUM-CHLORIDE COTRANSPORTER NUCLEIC ACIDS AND POLYPEPTIDES AND THERAPEUTIC AND SCREENING METHODS USING SAME

Cross Reference to Related Applications

This application is based on and claims priority to United States Provisional Application Serial Number 60/197,350, filed April 14, 2000, herein incorporated by reference in its entirety.

Grant Statement

This work was supported by National Institutes of Health grants DK02103, DK57708, and NS36758. Thus, the U.S. Government has certain rights in the invention.

Reference to Sequence Listing

The Sequence Listing associated with the instant disclosure has been submitted as an about 275 kilobyte file on compact disc (in duplicate). The enclosed compact discs are labeled to identify the applicants, the title of the invention, file name (1242-26-2.txt), creation date (April 15, 2001), computer system (IBM-PC/MS-DOS/MS-Windows) used to create the Sequence Listing, and attorney docket number (1242/26/2). The Sequence Listing submitted on compact disc is hereby incorporated by reference.

Technical Field

The present invention relates generally to isolated and purified proteins and nucleic acids. More particularly, the present invention relates to isolated and purified potassium-chloride cotransporter polypeptides and isolated and purified nucleic acids encoding the same.

Table of Abbreviations

ACCPN	peripheral neuropathy with or without agenesis of the corpus callosum
A-T	ataxia-telangiectasia
ATTC	American Tissue Type Collection
BSA	bovine serum albumin
CDR	complementarity determining region
CITB	California Institute of Technology BAC
CNPase	2', 3' –cyclic nucleotide 3' –phosphodiesterase
CNS	central nervous system
EMBL	European Molecular Biology Laboratory
EST	expressed sequence tag
HAT	cell culture media comprising hypoxanthine, aminopterin, and thymidine
hKCC2	human KCC2
hKCC3	human KCC3
hKCC4	human KCC4
IMAGE	integrated molecular analysis of genomes and their expression
KCC	potassium-chloride cotransporter; also "K^+-Cl^- cotransporter"
KCC3a	KCC3 isoform a
KCC3b	KCC3 isoform b
KCC3a2m	KCC3a lacking exon 2
KLH	keyhole limpet hemocyanin

mKCC3	mouse KCC3
mKCC4	mouse KCC4
ML-7	myosin light chain kinase inhibitor
mOsm	milli-osmole
NCC	sodium-chloride cotransporter; also A Na^{+}-Cl^{-} cotransporter"
NKCC1	Na^{+}-K^{+}-$2Cl^{-}$ cotransporter
NKCC2	Na^{+}-K^{+}-$2Cl^{-}$ cotransporter
NEM	N-ethylmaleimide
NIGMS	National Institute of General Medical Sciences
NKCC	sodium-potassium-chloride cotransporter; also A Na^{+}-K^{+}-$2Cl^{-}$ cotransporter"
NMDG	N-methyl-D-glucamine
NRSE	neuronal restricted silencing element
NRSF	neuronal restricted silencing factor
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PTZ	pentylenetetrazole
PKA	protein kinase A
PKC	protein kinase C
rabKCC1	rabbit KCC1
RACE	rapid amplification of cDNA ends
RT-PCR	reverse transcriptase polymerase chain reaction
STS	sequence-tagged site
TIGR	The Institute for Genome Research
TM	transmembrane domain
TMn	transmembrane domain n, where n is an integer
UTR	untranslated region
VNTR	variable number of tandem repeats
xKCC	*Xenopus* KCC

Background of the Invention

Cation-chloride cotransporters are typically categorized into one of three groups: Na^+-K^+-2 Cl^- cotransporters, Na^+-Cl^- cotransporter, and K^+-Cl^- cotransporters. Potassium-chloride cotransporters (K^+-Cl^- cotransporters or KCCs) were first described as a potassium efflux from erythrocytes that was induced by cell swelling. The cotransport of K^+ and Cl^- in erythrocytes is interdependent, with a 1:1 stoichiometry and low affinity constants for both ions. KCCs belong to a gene family of electroneutral cation-chloride cotransporters; *i.e.*, they are not influenced by membrane potential. Under most physiological conditions, they function as an efflux pathway that is involved in regulatory volume decrease. K^+-Cl^- cotransport is sensitive to the diuretics bumetanide and furosemide, but with much lower affinities than Na^+-K^+-2Cl^- cotransport.

Full-length cDNAs encoding two potassium-chloride cotransporters KCC1 and KCC2 have been reported in certain mammals. While human KCC1 has been cloned (Gillen et al. (1996) *J Biol Chem* 271(27):16237-16244; Holtzman et al. (1998) *Am J Physiol* 275(4 Pt 2):F550-564), KCC2 has not been cloned from in human beings. Both proteins exhibit a level of homology to other electroneutral cation-chloride cotransporters, including the bumetanide-sensitive Na^+- K^+-2 Cl^- cotransporters NKCC1 and NKCC2 (also known as BSC2 and BSC1, respectively), and the thiazide-sensitive Na^+- Cl^- cotransporter NCC (also known as TSC). An alternatively-spliced form of human KCC3 has also been reported. See Hiki et.al. (1999) *J Biol Chem* 274: 10661-10667.

International Publication Number WO 98/29431, published July 9, 1998, (inventors: Lifton et al.; assignee: Yale University) discloses roles for the human thiazide-sensitive Na-Cl transporter, TSC, the human ATP-sensitive potassium channel, ROMK, and the human Na^+-K^+-2Cl cotransporter, NKCC2, in causing a series of abnormal or pathological conditions. Amino acid and nucleotide sequences of several human wild-type and variant TSC, NKCC2 and ROMK proteins are disclosed.

U.S. Patent No. 4,895,807 issued January 23, 1990 to Cherksey discloses a purified membrane channel protein that was found to be related to both K^+ and Cl^- ion transport across cellular membranes. The channel protein is

described as having a molecular weight of approximately 280 to 300 kD, as determined, for example, by SDS-polyacrylamide gel electrophoresis.

International Publication Number WO 98/53067, published November 28, 1998 (inventors: Bevensee et al.; assignee Yale University) discloses the isolation and purification of polypeptides and nucleic acids pertaining to sodium bicarbonate cotransporters (NBCs).

International Publication Number WO 98/37198, published August 27, 1998 (inventors: Lal et al.; assignee: Incyte Pharmaceuticals Inc.) discloses a purified and isolated human sodium-dependent phosphate cotransporter (NAPTR) and purified and isolated polynucleotides encoding the same. The polypeptides and polynucleotides pertain functionally to the homeostasis of phosphate levels in the body.

International Publication Number WO 96/34288, published October 31, 1996 (inventors: Ni et al.; assignee: Eli Lilly and Company, Ltd.) discloses the isolation of a sodium-dependant inorganic phosphate cotransporter and a nucleic acid enclosing the same. The cotransporter was isolated from human brain tissue. The polypeptides and polynucleotides also pertain functionally to the homeostasis of phosphate levels in the body.

U.S. Patent No. 5,410,031 issued April 25, 1995 to Wright et al. (assignee University of California System) discloses a cDNA sequence encoding an amino acid sequence corresponding to mammalian Na^+/nucleoside cotransporter protein, abbreviated SNST. Thus, the encoded protein is a cotransporter that functions in conjunction with sodium ions and with nucleosides (*e.g.,* adenosine).

U.S. Patent No. 5,441,875 issued August 15, 1995 to Hediger (assignee: Brigham and Women's Hospital) discloses the isolation and purification of a urea transporter polypeptide and to a polynucleotide encoding the same. The disclosed polypeptide thus is described as functioning in the transmembrane transport of urea.

Potassium-chloride cotransport has been described for a number of cells, tissues, and organs, including blood, skin, heart, skeletal muscle, and brain. Potassium-chloride cotransport is thought to be involved in cell volume

regulation, trans-epithelial salt absorption, renal potassium secretion, and the regulation of both intra-and extra-cellular potassium and chloride ion concentrations.

Ion transporters have been implicated in a number of diseases, including hypertension, epilepsy, sickle cell anemia, Bartter's syndrome, and Meniere's disease. However, not all diseases currently thought to be associated with defective ion transport have been shown to be associated with known ion transport genes. Hence, there might be other ion transporter alleles that are defective or are affected in these diseases.

A major impediment to the study of K^+-Cl^- cotransport has also been the lack of specific high affinity inhibitors. Thus, further characterization of the molecular heterogeneity of potassium-chloride cotransporters has implications for the physiology and pathophysiology of a number of tissues. Additionally, the characterization of additional isoforms of KCCs would be particularly useful in screening for antibodies or pharmaceutical compositions which can modulate ion transport and hence provide treatments to ameliorate the effects of these diseases or disorders. Finally, the chromosomal localization and genomic characterization of the human KCC genes will be invaluable in the investigation of their role in monogenic disease, polygenic disease, and complex traits such as hypertension. Such characterization thus represents a long-felt and continuing need in the art.

Summary of the Invention

The present invention discloses isolated and purified polynucleotides encoding KCC genes, inclding mammalian KCC2, KCC3 and KCC4 genes, isolated and purified KCC polypeptides (including KCC2, KCC3 and KCC4 polypeptides), and the characterization of the role played by KCC polypeptides (including KCC2, KCC3 and KCC4 proteins) in modulating potassium transport. Preferably, a polypeptide of the invention is a recombinant polypeptide and comprises a mammalian KCC2, KCC3 or KCC4 polypeptide. More preferably, a polypeptide of the present invention comprises a human or mouse KCC2, KCC3 or KCC4 polypeptide. Most preferably, a polypeptide of the present

invention comprises a nucleotide or amino acid sequence selected from the sequences of any of SEQ ID NOs:1-16 or 112-113.

The present invention also provides an isolated and purified polynucleotide that encodes a KCC polypeptide (including a KCC2, KCC3 or KCC4 polypeptide) that modulates the levels of potassium and/or chloride as well as biological activities affected thereby. In a preferred embodiment, a polynucleotide of the present invention comprises a DNA molecule from a mammal. A preferred mammal is a mouse or a human. More preferably, a polynucleotide of the present invention encodes a polypeptide comprising an amino acid residue sequence of any of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, and 113. Most preferably, an isolated and purified polynucleotide of the invention comprises a nucleotide sequence of any of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and 112.

In another embodiment, the present invention provides an antibody immunoreactive with a KCC polypeptide (including a KCC2, KCC3 or KCC4 polypeptide) as described above. SEQ ID NOs:1-16 and 112-113 set forth nucleotide and amino acid sequences from exemplary mammals, mouse or human, and from a non-mammal vertebrate, *Xenopus laevis*. More preferably, the antibody of the invention is immunoreactive with a potassium-chloride cotransporter polypeptide comprising a human or mouse KCC2, KCC3 or KCC4 polypeptide. Even more preferably, an antibody of the invention is immunoreactive with a polypeptide comprising an amino acid residue sequence of any of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16 and 113. Also provided by the present invention are antibodies immunoreactive with homologs or biologically equivalent potassium-chloride cotransporter polynucleotides and polypeptides found in other vertebrates, and mammals. Optionally, an antibody of the invention is a monoclonal antibody.

In another aspect, the present invention provides a process of producing an antibody immunoreactive with a potassium-chloride cotransporter polypeptide as described above, the process comprising the steps of (a) transfecting a recombinant host cell with a polynucleotide that encodes a biologically active potassium-chloride cotransporter polypeptide; (b) culturing

the host cell under conditions sufficient for expression of the polypeptide; (c) recovering the polypeptide; and (d) preparing the antibody to the polypeptide. SEQ ID NOs:1-16 and 112-113 set forth nucleotide and amino acid sequences from representative mammals, human, mouse and *Xenopus*. Preferably, the host cell is transfected with a polynucleotide of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and 112. Even more preferably, the present invention provides an antibody prepared according to the process described above. Also contemplated by the present invention is the use of homologues or biologically equivalent KCC2, KCC3 and KCC4 polynucleotides and polypeptides found in other mammals to produce antibodies.

Alternatively, the present invention provides a process of detecting a potassium-chloride cotransporter polypeptide as described above, wherein the process comprises immunoreacting the polypeptide with an antibody prepared according to the process described above to form an antibody-polypeptide conjugate, and detecting the conjugate.

In yet another embodiment, the present invention provides a process of detecting a messenger RNA transcript that encodes a potassium-chloride cotransporter polypeptide as described above, wherein the process comprises hybridizing the messenger RNA transcript with a polynucleotide sequence that encodes that polypeptide to form a duplex; and detecting the duplex. Alternatively, the present invention provides a process of detecting a DNA molecule that encodes a potassium-chloride cotransporter polypeptide as described above, wherein the process comprises hybridizing DNA molecules with a polynucleotide that encodes a biologically active potassium-chloride cotransporter polypeptide to form a duplex; and detecting the duplex.

In another aspect, the present invention provides an assay or assay kit for detecting the presence of a potassium-chloride cotransporter polypeptide in a biological sample, where the kit comprises a first antibody capable of immunoreacting with a biologically active potassium-chloride cotransporter polypeptide. Preferably, the first antibody is present in an amount sufficient to perform at least one assay. Also preferably, an assay kit of the invention further comprises a second antibody that immunoreacts with the first antibody.

More preferably, the antibodies used in an assay kit of the present invention are monoclonal antibodies. Even more preferably, the first antibody is affixed to a solid support. More preferably still, the first and second antibodies comprise an indicator, and, preferably, the indicator is a radioactive label, a fluorescent label or an enzyme.

In an alternative aspect, the present invention provides an assay or assay kit for detecting the presence, in biological samples, of a potassium-chloride cotransporter polypeptide, the kits comprising a polynucleotide identical or complementary to a segment of at least 10 contiguous nucleotide bases of a polynucleotide that encodes a potassium-chloride cotransporter polypeptide.

In another embodiment, the present invention provides an assay or assay kit for detecting the presence, in a biological sample, of an antibody immunoreactive with a potassium-chloride cotransporter polypeptide, the kit comprising a first container containing a biologically active potassium-chloride cotransporter polypeptide that immunoreacts with the antibody, with the polypeptide present in an amount sufficient to perform at least one assay.

In still a further embodiment, this invention pertains to therapeutic methods based upon the modulation of the biological activity of potassium-chloride cotransporters via polynucleotides and polypeptides as described herein. Such therapeutic methods include gene therapy approaches using an isolated and purified polynucleotide of the present invention.

In another embodiment, the present invention provides genetic assays based on the genomic sequence of the human KCC2, KCC3, and KCC4 genes. The intronic sequence flanking the individual exons encoding the three genes is employed in the design of oligonucleotide primers suitable for the mutation analysis of human genomic DNA. Thus, intronic primers are used to screen for genetic variants by a number of PCR-based techniques, including single-strand conformation polymorphism (SSCP) analysis, SSCP/heteroduplex analysis, enzyme mismatch cleavage, and direct sequence analysis of amplified exons. Similar techniques can be applied to putative 5'-regulatory regions, *e.g.,* the putative promoters 5' of exons 1a and 1b of human KCC3. Automated methods

can also be applied the large-scale characterization of single nucleotide polymorphisms within and near the human KCC genes.

Once genetic variants have been detected in specific patient populations, *e.g.,* KCC3 mutations in patients with Andermann's syndrome, the present invention provides assays to detect the mutation by methods such as allele-specific hybridization, or restriction analysis of amplified genomic DNA containing the specific mutation. Again, these detection methods can be automated. In the case of genetic disease or human phenotypes caused by repeat expansion, the present invention provides an assay based on PCR of genomic DNA with oligonucleotide primers flanking the involved repeat.

In yet another aspect, the present invention provides a transgenic animal. In one embodiment of the present invention, the transgenic animal can comprise a mouse with targeted modification of the mouse KCC2, KCC3, and KCC4 genes and can further comprise mice strains with complete or partial functional inactivation of the KCC genes in all somatic cells. In an alternative embodiment, a transgenic animal in accordance with the present invention is prepared using anti-sense or ribozyme KCC constructs, driven by a universal or tissue-specific promoter, to reduce levels of individual KCCs in somatic cells, thus achieving a "knock-down" of individual isoforms. The present invention also provides the generation of murine strains with conditional or inducible inactivation of individual or multiple KCC genes.

The present invention also provides mice strains with specific "knocked-in" modifications in the KCC2, KCC3, or KCC4 genes. This includes mice with genetically and/or functionally relevant point mutations in the KCC genes, in addition to manipulations such as the insertion of disease-specific repeat expansions.

Thus, a key aspect of this invention pertains to the discovery of novel potassium-chloride cotransporter polypeptides and nucleic acids. Preferred nucleic acid and amino acid sequences are described in SEQ ID NOs:1-16 adn 112-113. It is thus another aspect of this invention to provide a purified and isolated potassium-chloride cotransporter polypeptide having a role in the biological activity of potassium-chloride concentration modulation.

The foregoing aspects and embodiments have broad utility given the biological significance of the potassium-chloride cotransporter proteins. By way of example, the foregoing aspects and embodiments are useful in the preparation of screening assays and assay kits that are used to identify compounds that affect or modulate potassium-chloride cotransporter biological activity, or that are used to detect the presence of the proteins and nucleic acids of this invention in biological samples.

Some of the aspects and objects of the invention having been stated herein above, other aspects and objects will become evident as the description proceeds, when taken in connection with the accompanying Drawings and Examples as best described herein below.

Brief Description of the Drawings

Figure 1 is a schematic representation of the cDNAs encoding human KCC2, human, mouse and *Xenopus* KCC3 and KCC4. A full-length cDNA is shown for each isoform; coding sequence is boxed and solid lines represent 5'- and 3'-UTR. The relative positions of partial cDNAs, derived from EST cDNA clones, RT-PCR, 5'-RACE RT-PCR, and library screening, are shown as shaded rectangles below each full-length sequence. The IMAGE or TIGR clone numbers are displayed below individual EST cDNAs. The mouse KCC3a and KCC3b cDNAs were obtained by sequential RT-PCR of murine tissues, using human KCC3a and KCC3b primer pairs. The extreme 5' end of the KCC3a and KCC3b coding sequences were then determined by sequence analysis of murine exons 1a and 1b, and the 3'-UTR of was obtained from a mouse EST cDNA. In the case of hKCC2, the 5' end of the cDNA was cloned by RT-PCR, and the two 3' coding sequence ESTs (363600 and 362310) overlap at a Not I site (nucleotide 3322 in the full-length cDNA). The figure is drawn to the scale indicated.

Figure 2A is a schematic diagram depicting the genomic structure of human KCC3. Boxes represent exon sequences, numbered as indicated, and the line represents 5' UTR, 3' UTR, and intronic regions. KCC3 has two separate first coding exons, denoted 1a and 1b. 5'-RACE PCR of mouse

kidney has mapped the transcriptional start site of mKCC3b just 5' of the start codon in exon 1b, hence the two isoforms are generated by transcriptional initiation at two separate promoters. Human exon 1a has a 5'-flanking CpG island (GenBank/EMBL Accession No. Z63283). The sequences for the putative mouse KCC3 exon 1a and 1b promoters are included in SEQ ID NOs:18 and 19, and that of human KCC3 exon 1a is included in SEQ ID NO:17. The figure is drawn to the scale indicated.

Figure 2B is a schematic diagram depicting the genomic structure of exons 2-24 of human KCC4. Open boxes represent exon sequences, numbered as indicated, and the line represents 5' UTR, 3' UTR, and intronic regions. The polymorphic VNTR (variable number of tandem repeat; shaded rectangle) marker D5S110, contained in the genomic subclone pMS621, is found in the intron between exons 17 and 18 of hKCC4. The figure is drawn to the scale indicated.

Figure 3 is a map showing the structure of an 18-kb mouse genomic clone encoding the KCC2 gene. Magnification of exon 1 displays the position corresponding to the 5' end of the rat cDNA, the 5' untranslated region (UTR), the ATG start of the KCC2 protein (origin of arrow, M, methionine; L, leucine; N, asparagine), and the exon/intron boundary (5' splice junction). The position of the KCC2 NRSE sequence, depicted as a star, is observed downstream of exon 1. The alignment of the KCC2 putative neuronal-restrictive silencing element (NRSE) sequence with the NRSE consensus sequence is shown. Identical residues are boxed and residues that are likely subject to modifications are marked with an asterisk. M and S in the consensus sequence represent (A or C) and (C or G), respectively. Selected restrictions sites are shown as landmarks: X, *Xho*I; E, *EcoR*I. The thick black line starting at the *EcoR* I site indicates the fragment which was sequenced, and the thick gray line represents a 10.2-kb genomic fragment released in Genbank under Accession No. AJ011033. The genomic clone is drawn to a scale indicated by the 1 kb bar, and the magnification of exon 1 is drawn to a scale indicated by the 20 bp bar.

Figures 4A and 4B are autoradiographs of an electrophoretic mobility shift assay (EMSA) depicting the mKCC2 NSRE bound to nuclear proteins from a murine neural progenitor cell line, performed as described in the Examples.

Figure 4A is an autoradiograph of an EMSA showing a retarded band in reactions where nuclear proteins were incubated with the probe, indicating an interaction between the DNA probe and a nuclear protein.

Figure 4B is an autoradiograph of an EMSA performed using unlabeled (cold) NRSE DNA or unrelated DNA. A protein-DNA complex, evidenced by a retarded band (arrowhead labeled "complex"), was still present when excess cold DNA of unrelated sequence (unspecific oligo) was added to the reaction. By contrast, excess cold NSRE DNA can compete with the NSRE probe, indicated by the absence of the labeled protein-DNA complex.

Figure 5 is a bar graph depicting transcriptional repression conferred by the mKCC2 NSRE. Expression of the indicated reporter constructs was assayed in non-neuronal C17 cells. Relative luciferase activities were measured with promoterless PGL3-basic vector (open bar), PGL3 vector containing 1,500 base pairs of 5' flanking region of the KCC2 gene (EcoRI-XhoI fragment) (black bar), and PGL3 vector including both the KCC2 promoter and the 21-base pair NRSE element (gray bar). Significant luciferase activity is generated by the KCC2 promoter ($P < 0.01$), and KCC2-induced luciferase activity is completely inhibited by the presence of the NRSE element. Each bar represents the mean ± standard error of 3 measurements. Experiments shown in this figure were repeated 4 times with similar results.

Figure 6 is a bar graph that summarizes the baseline functional characterization of KCC1and KCC4 expressed in *Xenopus* laevis oocytes. *Xenopus* oocytes were each injected with water (H_20) or with cRNA encoding rabKCC1 or mKCC4, as indicated. K^+-Cl^- cotransport was assayed by measuring chloride-dependent uptake of $^{86}Rb^+$, a surrogate for K^+, as described in Example 12. Uptakes were performed under both isotonic (200 mOsm/kg) and hypotonic (100 mOsm/kg) conditions in the presence (open bars) or absence (black bars) of extracellular chloride. Each bar represents a mean of data collected from 90 oocytes extracted from five animals. $^{86}Rb^+$ uptake was

measured during a 60-minute interval. The asterisk (*) indicates that $^{86}Rb^+$ uptake in *Xenopus* oocytes expressing mKCC4 and in the presence of extracellular chloride is significantly increased when compared with $^{86}Rb^+$ uptake in control oocytes ($p < 0.01$).

Figure 7A shows the functional expression of a K^+- Cl^- cotransporter in *Xenopus* laevis oocytes that were injected with water or with 25 ng of hKCC2 cRNA, as indicated. $^{86}Rb^+$ uptake assays were performed in isotonic solutions of 210 mOsm/kg, in the presence (open bars) or absence (black bars) of extracellular Cl^-. The uptake solutions contained 10 mM K^+ and 50 mM Cl^-. Each bar represents a mean of data obtained from 40-60 oocytes extracted from five different frogs. $^{86}Rb^+$ uptake was performed for 60 minutes. The asterisk (*) denotes a significant difference in $^{86}Rb^+$ uptake relative to $^{86}Rb^+$ uptake observed in water-injected oocytes in the presence of extracellular Cl^- ($p<0.001$); the cross symbol (†) denotes significantly increased uptake in the KCC2 group in the presence of chloride ($p<0.001$) when compared to water-injected oocytes in the absence of extracellular Cl^-. $^{86}Rb^+$ uptake values indicate the mean +/- standard error. H_20/open bars, activity of xKCC (*Xenopus* K^+- Cl^- cotransporter) at 210 mOsm/kg = 145 ± 13; H_20/black bars, activity of xKCC at 210 mOsm/kg in the absence of Cl^- = 69.3 ± 6.7; hKCC2/open bars, activity of KCC2 at 210 mOsm/kg = 434 ± 31; hKCC2/black bars, activity of hKCC2 at 210 mOsm/kg in the absence of Cl^- = 119 ± 8.5.

Figure 7B shows the functional expression of a K^+- Cl^- cotransporter in *Xenopus* laevis oocytes that were injected with water or with 25 ng of cRNA from hKCC2, as indicated. $^{86}Rb^+$ uptake assays were performed in hypotonic solutions with 120 mOsm/kg, in the presence (open bars) or absence (black bars) of extracellular Cl^-. The uptake solutions contained 10 mM K^+ and 50 mM Cl^-. Each bar represents a mean of data obtained from 40-60 oocytes extracted from five different frogs. $^{86}Rb^+$ uptake was performed for 60 minutes. The asterisk (*) denotes a significant difference from uptake in the H2O control group ($p<0.001$); the cross symbol (†) denotes a significant difference from uptake in the hKCC2 group in the presence of chloride ($p<0.001$). $^{86}Rb^+$ uptake values indicate the mean +/- standard error. H_20/open bars, activity of xKCC at

120 mOsm/kg = 221 ± 11; H_20/black bars, activity of xKCC at 120 mOsm/kg in the absence of Cl^- = 71.5 ± 6.6; hKCC2/open bars, activity of hKCC2 at 120 mOsm/kg = 8713 ± 545; hKCC2/black bars, activity of hKCC2 at 120 mOsm/kg in the absence of Cl^- = 430 ± 50.

Figure 8 presents bar graphs that compare K^+-Cl^- cotransport mediated by xKCC, hKCC2 and mKCC4 under isotonic (210 mOsm/kg) and hypotonic (120 mOsm/kg) conditions. hKCC2 and mKCC4, which are ~70% identical, exhibit dramatic differences in their response to cell volume. Like the other KCCs, however, swelling activation of hKCC2 depends on dephosphorylation mediated by serine-threonine protein phosphatases, although baseline isotonic activity does not. KCC2 is unique among the four KCCs in mediating significant K^+-Cl^- cotransport under isotonic conditions. Open bars, uptake assays performed in control medium containing 10 mM K^+ and 50 mM Cl^-; black bars, uptake assays performed in the absence of extracellular chloride.

Figure 9A is a line graph that summarizes a kinetic characterization of $^{86}Rb^+$ uptake in response to extracellular K^+ concentration in *Xenopus* oocytes injected with mKCC4 cRNA.

Figure 9B is a line graph that summarizes a kinetic characterization of $^{86}Rb^+$ uptake in response to extracellular Cl^- concentration in *Xenopus* oocytes injected with mKCC4 cRNA.

Figure 9C is a line graph that summarizes a kinetic characterization of $^{86}Rb^+$ uptake in response to extracellular K^+ concentration in *Xenopus* oocytes injected with rabKCC1 cRNA.

Figure 9D is a line graph that summarizes a kinetic characterization of $^{86}Rb^+$ uptake in response to extracellular Cl^- concentration in *Xenopus* oocytes injected with rabKCC1 cRNA.

Figure 10 presents line graphs depicting concentration dependence of K^+-Cl^- cotransport mediated by hKCC2. Uptake assays were simultaneously assessed in water-injected oocytes. To determine $^{86}Rb^+$ uptake due to hKCC2, the mean values for water groups were subtracted from the mean values for corresponding hKCC2 groups.

Figure 11 presents bar graphs depicting differences in the slope of activation of hKCC3a and hKCC3b by cell swelling, but that both isoforms begin to activate at an extracellular osmolarity of 160 mOsm/kg. These differences in activation can be attributable to differences between predicted phosphorylation of their divergent amino termini (Mount et al. (1999) *J Biol Chem* 274:16355-16362).

Figure 12 is a set of bar graphs depicting the anion dependence of rabKCC1, hKCC3a, and mKCC4. $^{86}Rb^+$ influx was assessed in hypotonic uptake medium containing 40 mM NMDG gluconate plus 10 mM concentration of KCl (control group) or 10 mM potassium salts of each of the anion substitutes (KBr, KH_2PO4, KI, potassium gluconate, and KSCN). Preincubation was done in a solution containing 50 mM NMDG gluconate. Data was normalized by designating $^{86}Rb^+$ uptake in the control group as 100%. Each bar represents the mean ± standard error determined in at least 15 oocytes. The observed differences in anion selectivity among rabKCC1, hKCC3a, and mKCC4 are likely due to variation with TM4 and TM7, segments thought to confer anion affinity on the cation-chloride cotransporters. Asterisk (*) indicates significantly increased $^{86}Rb^+$ uptake when compared to uptake observed for the conrol group.

Figure 13 is a bar graph depicting the anion dependence of K^+ - Cl^- cotransport mediated by hKCC2, *i.e.* the relative activity of the transporter in the presence of anions other than chloride. $^{86}Rb^+$ influx was assessed in hKCC2 injected oocytes using hypotonic uptake mediums containing 40 mM NMDG-gluconate plus 10 mM concentration of KCl (as control group) or 10 mM concentration of any of the anion substitutes (KBr, KH_2PO4, KI, K-gluconate and KSCN). Asterisk (*) indicates significantly increased $^{86}Rb^+$ uptake when compared to uptake observed for the conrol group ($p < 0.01$).

Figure 14 presents line graphs depicting the effect of extracellular K^+ concentration ($[K^+]_e$) on the inhibition of $^{86}Rb^+$ uptake by the loop diuretics furosemide and bumetanide. Data from mKCC4-injected oocytes are presented in the upper panels, and data from rabKCC1-injected oocytes are shown in the lower panels. In all experiments, the Cl^- concentration in the

extracellular medium was 50 mM, whereas the extracellular potassium concentration was varied from 2 – 20 mM. The control group for each KCC comprised oocytes not treated with diuretic, and the mean uptake in the absence of loop diuretic for each KCC was designated as 100% uptake. Data from diuretic-treated groups was normalized to the uptake in the control group. Experimental groups were exposed to 2 mM furosemide or bumetanide during the incubation and uptake periods. Each point represents the mean ± standard error of data obtained from at least 15 oocytes.

Figure 15 presents line graphs depicting concentration-response profiles for inhibition of KCC4- and KCC1-mediated cation-chloride transport by furosemide and bumetanide. Groups of 15 *Xenopus* ooctyes microinjected with mKCC4 or rabKCC1 were exposed to 20-2000 μM furosemide or bumetanide in the preincubation and uptake media. Oocytes not exposed to loop diuretics was designated the control group, and the percentage influx observed in this group was designated as 100%. Data were normalized as the percentage of influx relative to the control group. Each point represents the mean ± standard error of data obtained from at least 15 oocytes. (○) /solid line, oocytes injected with mKCC4; (□) /dashed line oocytes injected with rabKCC1.

Figure 16 is a line graph depicting dose-dependent inhibition of hKCC2-mediated cation-chloride cotransport by furosemide or bumetanide. Groups of oocytes microinjected with hKCC2 cRNA were exposed to increasing concentrations of furosemide or bumetanide in the preincubation and uptake mediums, from 2 to 2000 μM. Uptake assays were performed in hypotonic conditions. Each point represents the mean ± standard error of at least 15 oocytes. (□) / dashed line, furosemide treatment; (○) /solid line, bumetanide treatment.

Figure 17 presents bar graphs depicting hKCC3b-mediated K^+-Cl^- cotransport in *Xenopus* oocytes, with minimal activity under isotonic conditions (220 mOsm/kg) and marked activation under hypotonic conditions (110 mOsm/kg). Open bars, uptake assays performed in a control medium containing 10 mM K+ and 50 mM Cl^- ; black bars, uptake assays performed in

medium lacking Cl^- ; gray bars, uptake assays performed in medium containing 2 mM furosemide. Asterisks (*) indicate a significant difference in $^{86}Rb^+$ uptake when compared to uptake in the corresponding control group ($p < 0/01$).

Figure 18A is a bar graph depicting the effect of the inhibitor DIDS on $^{86}Rb^+$ uptake in *Xenopus* oocytes microinjected with mKCC4, hKCC3, or rabKCC1 and incubated in hypotonic conditions (120 mOsm/kg), in the presence of 2 mM extracellular K^+, 50 mM Cl^- (open bars) or 50 mM extracellular K^+, 50 mM Cl^- (hatched bars). $^{86}Rb^+$ uptake was assessed in control groups in the absence of DIDS. Experimental groups were exposed to 100 μM DIDS during the incubation and uptake periods. Each bar represents the mean ± standard error of data obtained from at least 15 oocytes.

Figure 18B is a bar graph depicting the effect of the inhibitor DIOA on $^{86}Rb^+$ uptake in *Xenopus* oocytes microinjected with mKCC4, hKCC3, or rabKCC1 and incubated in hypotonic conditions (120 mOsm/kg), in the presence of 2 mM extracellular K^+, 50 mM Cl^- (open bars) or 50 mM extracellular K^+, 50 mM Cl^- (hatched bars). $^{86}Rb^+$ uptake was assessed in control groups in the absence of DIOA. Experimental groups were exposed to 100 μM DIOA during the incubation and uptake periods. Each bar represents the mean ± standard error of data obtained from at least 15 oocytes.

Figure 19 presents bar graphs depicting the effect of 10 mM $BaCl_2$ on $^{86}Rb^+$ uptake induced by microinjection of oocytes with *Xenopus* KCC (KCCx), rabKCC1, hKCC3, or mKCC4 cRNA. $^{86}Rb^+$ uptake in the control groups (open bars) was measured using a hypotonic uptake medium containing 40 mM NMDG chloride and 10 mM KCl. $^{86}Rb^+$ uptake in oocytes treated with $BaCl_2$ (black bars) was measured using a hypotonic medium containing 30 mM NMDG chloride, 10 mM $BaCl_2$, and 10 mM KCl. Each bar represents a mean of data obtained from 20 oocytes. Asterisks (*) indicate $^{86}Rb^+$ uptake values of the $BaCl_2$-treated oocytes that are significantly different when compared to $^{86}Rb^+$ uptake in control groups ($p < 0.01$). KCC4 in particular is partially sensitive to 10 mM barium, consistent with the localization of KCC4 at the basolateral membrane of thick ascending limb cells.

Figures 20A-20D present bar graphs depicting the effect of 1 mM NEM on $^{86}Rb^+$ uptake in mKCC4-injected and rabKCC1-injected oocytes in isotonic and hypotonic conditions.

Figure 20A is a bar graph depicting $^{86}Rb^+$ uptake in mKCC4-injected oocytes in isotonic medium. Each bar represents the mean ± standard error of data obtained from 20 oocytes. Open bars, $^{86}Rb^+$ uptake in control conditions lacking NEM; black bars, $^{86}Rb^+$ uptake in the absence of extracellular Cl^-; hatched bars $^{86}Rb^+$ uptake in the presence of NEM. The asterisk (*) indicates that $^{86}Rb^+$ uptake in NEM-treated oocytes is significantly increased relative to the control group ($p < 0.0001$).

Figure 20B is a bar graph depicting $^{86}Rb^+$ uptake in rabKCC1-injected oocytes in isotonic medium. Each bar represents the mean ± standard error of data obtained from 20 oocytes. Open bars, $^{86}Rb^+$ uptake in control conditions lacking NEM; black bars, $^{86}Rb^+$ uptake in the absence of extracellular Cl^-; hatched bars $^{86}Rb^+$ uptake in the presence of NEM. The asterisk (*) indicates that $^{86}Rb^+$ uptake in NEM-treated oocytes is significantly increased relative to the control group ($p < 0.0001$).

Figure 20C is a bar graph depicting $^{86}Rb^+$ uptake in mKCC4-injected oocytes in hypotonic medium. Each bar represents the mean ± standard error of data obtained from 20 oocytes. Open bars, $^{86}Rb^+$ uptake in control conditions lacking NEM; black bars, $^{86}Rb^+$ uptake in the absence of extracellular Cl^-; hatched bars $^{86}Rb^+$ uptake in the presence of NEM. The asterisk (*) indicates that $^{86}Rb^+$ uptake in NEM-treated oocytes is significantly decreased relative to the control group ($p < 0.0001$).

Figure 20D is a bar graph depicting $^{86}Rb^+$ uptake in rabKCC1-injected oocytes in hypotonic medium. Each bar represents the mean ± standard error of data obtained from 20 oocytes. Open bars, $^{86}Rb^+$ uptake in control conditions lacking NEM; black bars, $^{86}Rb^+$ uptake in the absence of extracellular Cl^-; hatched bars $^{86}Rb^+$ uptake in the presence of NEM. The asterisk (*)

indicates that $^{86}Rb^+$ uptake in NEM-treated oocytes is significantly decreased relative to the control group ($p < 0.0001$).

Figure 21 presents bar graphs depicting differential response of the KCCs to 50 µM mercury ($HgCl_2$). rabKCC1 and hKCC3a activated by hypotonic conditions and are resistant to 50 µM mercury $HgCl_2$, whereas hKCC2 and in particular mKCC4 are sensitive to this agent. Open bars, uptake assays performed in control medium containing 10 mM K^+ and 50 mM Cl^- ; black bars, uptake assays performed in medium containing 50 µM mercury $HgCl_2$. Asterisk (*) indicates a significant reduction in uptake activity relative to the control group ($p < 0.001$).

Figure 22 presents bar graphs depicting the differential response of native and mutant mKCC4 polypeptides to $HgCl_2$. A quadruple mutant mKCC4 maintains some sensitivity to $HgCl_2$, whereas the quintuple mKCC4 mutant is resistant. Quadruple, mKCC4 mutant with four cysteines mutated (C256A/C469N/C565A/C647M); Quintuple, mKCC4 mutant with five cysteine residues mutated (C256A/C469N/C565A/C647M/C633S); open bars, uptake assays performed in control medium containing 10 mM K^+ and 50 mM Cl^- ; black bars, uptake assays performed in medium lacking Cl^-; hatched bars, ssuptake assays performed in medium containing $HgCl_2$. Asterisk (*) indicates a significant reduction in KCC4 activity ($p < 0.01$).

Figure 23 is a bar graph depicting the effect of the myosin light chain kinase inhibitor ML-7 on hKCC3a activity in *Xenopus* oocytes. K^+ - Cl^- cotransport is activated by 100 µM ML-7 (989 ± 115 pmol/oocyte/hour for KCC3a in the absence of ML-7 versus 1698 ± 298 pmol/oocyte/hour in the presence of ML-7 at 160 mOsm/kg). Black bars from left to right, $^{86}Rb^+$ uptake by xKCC in the presence of Cl^-, $^{86}Rb^+$ uptake by xKCC in the presence of Cl^- and ML-7, $^{86}Rb^+$ uptake by hKCC3a in the presence of Cl^-, $^{86}Rb^+$ uptake by hKCC3a in the presence of Cl^- and ML-7; gray bars from left to right, $^{86}Rb^+$ uptake by xKCC in the absence of Cl^-, $^{86}Rb^+$ uptake by xKCC in the absence of Cl^- and in the presence of ML-7, $^{86}Rb^+$ uptake by hKCC3a in the absence of Cl^-, $^{86}Rb^+$ uptake by hKCC3a in the absence of Cl^- and in the presence of ML-7

Figure 24 presents bar graphs depicting the effect of the protein phosphatase inhibitors calyculin A, okadaic acid, and cypermethrin upon the swelling-induced activation of mKCC4 or rabKCC1. White bar, $^{86}Rb^+$ influx in control oocytes not treated with inhibitor in hypotonic medium; hatched bar, $^{86}Rb^+$ influx in oocytes treated with 100 nM calyculin A in hypotonic medium; black bar, $^{86}Rb^+$ influx in oocytes treated with 1 nM okadaic acid in hypotonic medium; gray bar, $^{86}Rb^+$ influx in oocytes treated with 100 pM cypermethrin in hypotonic medium. Each bar represents the mean ± standard error observed in at least 15 oocytes.

Figure 25A is a bar graph depicting the effect of the protein phosphatase inhibitor calyculin A (100 nM) on the isotonic K^+-Cl^- cotransport mediated by hKCC2. $^{86}Rb^+$ influx was assessed in a control group (white bars), in the absence of extracellular Cl^- (black bars), or in the presence 100 mM calyculin (hatched bars). Each bar represents the mean ± standard error of data obtained using at least 15 oocytes. Asterisk (*) indicates that $^{86}Rb^+$ influx was significantly reduced in the absence of Cl^- relative to the control group.

Figure 25B is a bar graph depicting the effect of shows the effect of the protein phosphatase inhibitor calyculin A (100 nM) on the swelling-induced K^+Cl^- cotransport mediated by hKCC2. $^{86}Rb^+$ influx was assessed in a control group (white bars), in the absence of extracellular Cl^- (black bars), or in the presence 100 mM calyculin (hatched bars). Each bar represents the mean ± standard error of data obtained using at least 15 oocytes. Asterisk (*) indicates that $^{86}Rb^+$ influx was significantly reduced in the absence of Cl^-, or in the combined absence of Cl^- and presence of calyculin, relative to the control group.

Figure 26 is a bar graph depicting hKCC3b-mediated K^+-Cl^- cotransport in *Xenopus* oocytes microinjected with H20 or hKCC3b cRNA as indicated, in the presence of putative inhibitors. hKCC3b-mediated K^+-Cl^- cotransport is sensitive to furosemide treatment and blocked by calyculin A treatment. Open bars, uptake assays performed in a control medium containing 10 mM K^+ and 50 mM Cl^-; hatched bars, uptake assays performed in medium lacking Cl^-; bars with horizontal stripes, uptake assays performed in the presence of 100 nM

calyculin A; black bars, uptake assays performed in the presence of okadaic acid; gray bars, uptake assays performed in the presence of cypermetrin.

Figure 27A is an autoradiograph of a Northern blot showing that human KCC2 is heavily induced during the *in vitro* differentiation of human NT2 teratocarcinoma cells, which do not express this transporter in the undifferentiated state, into "NT2-N" neuronal cells. NT2-N cells are used as a neuronal cell model, and are known to express several subtypes of $GABA_A$ receptors as well as multiple neurotrophin receptors. As such these cells provide a cell model for understanding the function, post-transcriptional regulation, and transcriptional regulation of human KCC2.

Figure 27B is an autoradiograph of a Northern blot of mouse tissues probed with both a KCC3 exon 1a-specific and a KCC3 exon 1b-specific probe. KCC3a is particularly abundant in brain and muscle, whereas KCC3b is most abundant in kidney, indicating that the two promoters are differentially regulated.

Figure 27C is a photograph of RT-PCR of mouse tissues with a primer in KCC3 exons 1a and 4. Two bands are amplified, corresponding to alternative splicing of exon 2; the shorter band corresponds to isoforms lacking this 45 base-pair cassette exon. Sequence data for mouse and human KCC3a lacking exon 2 (KCC3a-2m) are included in SEQ ID NOs:3-6.

Figure 27D is an autoradiograph of a Western blot using the amino-terminal KCC3-specific antibody, generated to a peptide antigen from exon 3 (KKARNAYLNNSNYEEGDEY; SEQ ID NO:116). Although immunoreactivity has not been tested against the five KCCs heterologously expressed in *Xenopus* oocytes, the peptide antigen is not found in the KCC1, KCC2 or KCC4 sequences, and does not detect other proteins in stringent BLAST searches of the non-redundant and EST databases. The KCC3 antibody reacts with proteins of ~160 kDa in several tissues, including brain and kidney (shown in Figure 27D for renal cortex and outer medulla (OM), as well as brain). This reactivity is abolished when antibody is pre-incubated with peptide antigen ("IAB" sample, shown for renal cortex in Figure 27D).

Figure 27E is an autoradiograph of a Western blot using the amino-terminal KCC3-specific antibody, generated to a peptide antigen from exon 3 (KKARNAYLNNSNYEEGDEY; SEQ ID NO:116). A KCC3 protein is detected in membrane samples from murine red cells (RBC lane), and from a mouse proximal tubule cell line (tsMPT lane) and human umbilical vein endothelial cells (HUVEC lane). The presence of KCC3 in red cells suggests that KCC3 contributes to red cell K^{+}-Cl^{-} cotransport activity.

Figure 27F is an autoradiograph of a Western blot showing that an amino-terminal KCC4-specific antibody, generated to a peptide antigen from exon 1 (AERTEEPESPESVDQTSP; SEQ ID NO:117), detects a broad band of proteins between 160 and 180 kDa in molecular mass.

Figure 27G is an autoradiograph of a Western blot prepared using mouse tissues showing that for an amino-terminal KCC4-specific antibody, generated to a peptide antigen from exon 1 (AERTEEPESPESVDQTSP; SEQ ID NO:117), two separate bands can be resolved in renal cortex and outer medulla (OM), with the lower mass band predominant in cortex and the higher mass band predominant in outer medulla. This heterogeneity might be due to differential glycosylation, but is more likely due to alternative splicing.

Figure 27H is an autoradiograph of a Western blot prepared using mouse tissues showing that for an amino-terminal KCC4-specific antibody, generated to a peptide antigen from exon 1 (AERTEEPESPESVDQTSP; SEQ ID NO:117), reactivity is abolished by immunoabsorption with peptide antigen.

Figure 27 I is an autoradiograph of a Western blot of total protein from *Xenopus* oocytes injected with water, or singly injected with cRNA (25 ng/oocyte) encoding rabKCC1, hKCC2, hKCC3a, or mKCC4. C-terminal fusion protein-specific antibodies to KCC1 and KCC2 served as positive controls for the expression of KCC1 and KCC2. The KCC3 exon 3 antibody and the KCC4 exon 1 antibody are specific for their respective isoforms.

Figure 27J is a photograph depicting immunofluorescence of KCC3 in mouse proximal tubules of both wildtype mice (+/+) and mice homozygous for a targeted deletion of exon 3 of mKCC3 (-/-) (KCC3 1: 4000, Alexa 594 1-5,000).

The presence of KCC3 is observed as a light gray signal. Reactivity is lost in the knockout mice, demonstrating the specificity of this antibody.

Figure 28 is an autoradiograph of a Western blot prepared using proteins derived from mouse brain. An anti-KCC3 antibody was used to detect KCC3. Microsomal protein from mouse brain (70 g/lane) was separated by 7.5% SDS-PAGE, transferred, and probed with preimmune serum (1:300), immune serum (1:300), purified antibody (1:1000) and purified antibody preabsorbed with immune peptide. Specific KCC3 signal consisting of a protein doublet (155-165 kDa, lane 3) is also seen in immune serum but not in preimmune serum, nor in purified antibody preabsorbed with antigenic peptide.

Figure 29 is a Northern blot prepared using total RNA (10 μg/lane) isolated from different regions of mouse brain. The membrane was probed successively with KCC3 1a-specific, KCC3 1b-specific, and actin probes. KCC3b was not detected at all, but KCC3a was present in all regions of the brain examined. Densitometry of KCC3a and actin was performed, and densitometry rations of KCC3a/actin were calculated. Equivalent amounts of KCC3a transcripts were measured in all regions of the brain, with the exception of the choroid plexus, which expressed one-third of the amount of other regions.

Figure 30A is a Western blot prepared from mouse and rat microsomal protein samples (70 μg/lane) derived from the indicated brain regions. An anti-KCC3 polyclonal antibody was used to detect KCC3 protein. A protein doublet is identified in whole brain, cerebral cortex, hippocampus, diencephalon, brainstem and cerebellum. Expression was lower in the hippocampus in two of three western blot analyses.

Figure 30B is a Western blot prepared from mouse and rat microsomal protein samples derived from the indicated brain regions. An anti-KCC3 polyclonal antibody was used to detect KCC3 protein. Relatively high levels of KCC3 are detected in white matter tracts and the spinal cord, and lower levels are detected in the dorsal root ganglia and peripheral nerve.

Figure 30C is a Western blot prepared from crude membrane samples derived from rat brains at the indicated developmental stages. P1, postnatal

day 1; P7, postnatal day 7; P14, postnatal day 14; P21, postnatal day 21; Ad., adult brains (> postnatal day 60). Numbers listed below each lane indicate abundance of KCC3 relative to KCC3 levels in P1 brain, as measured by densitometry.

Figure 31A is a fluorescence micrograph of adult mouse spinal cord stained using an anti-KCC3 polyclonal antibody. The presence of KCC3 is observed as regions of light gray signal. KCC3 is detected in the dorsal columns, which consist of highly myelinated axonal tracts. Scale bar = 100 µm.

Figure 31B is a fluorescence micrograph of the same spinal cord section depicted in Figure 31A stained using the oligodendrocyte marker anti-CNPase antibody. The presence of CNPase is observed as regions of light gray signal.

Figure 31C is a merged image of Figures 32A and 32B, depicting co-staining of KCC3 and CNPase in the dorsal column. Co-staining of KCC3 and CNPase is observed as regions of white signal, representing the combined light gray signals shown in Figures 32A and 32B.

Figure 31D is a fluorescent micrograph of an adult ventral spinal cord stained using an anti-KCC3 antibody. KCC3 is detected in the ventral white matter tracts and is observed as a light gray signal. In gray matter, KCC3 is detected only in crossing fibers.

Figure 31E is a fluorescent micrograph of the same ventral spinal cord section depicted in Figure 17D stained using an anti-CNPase antibody. The presence of CNPase is observed as a light gray signal. CNPase is also detected in the white matter region of the ventral spinal cord, indicating co-localization of KCC3 and CNPase in the anterolateral column. Scale bar = 100 µm.

Figure 31F is a fluorescence micrograph of an adult spinal cord stained with anti-KCC3 and anti-CNPase antibodies that were immunoabsorbed with the corresponding specific antigen prior to incubation with the tissue. KCC3 and CNPase are not detected.

Figure 32A is a fluorescence micrograph of a coronal section of the forebrain labeled with an anti-KCC3 antibody. The presence of KCC3 is detected as regions having a light gray signal. KCC3 is detected in the white

matter tract of the corpus callosum, as well as in the CA1 neuronal layer of the hippocampus. Arrows point to KCC3-positive cell bodies in the vicinity of the white matter tract. Scale bar = 50 μm.

Figure 32B is a fluorescence micrograph of the same region of the forebrain depicted in Figure 34A stained using the oligodendrocyte (myelin) marker anti-CNPase. The presence of CNPase is observed as regions having a light gray signal. CNPase is detected in the white matter tract and is absent from the CA1 layer of the hippocampus.

Figure 32C is a merged image of Figures 34A and 34B. Co-localization of KCC3 and CNPase is observed as regions of white signal, representing the combined summation of overlapping regions of gray signal. KCC3 and CNPase are co-localized in the corpus callosum. However, numerous cell bodies are KCC3-positive but devoid of CNPase signal (arrows).

Figure 32D is a fluorescence micrograph depicting dentate gyrus labeled with an anti-KCC3 antibody. Numerous granular cell bodies are labeled and are observed as regions of light gray signal. Scale bar = 50 μm.

Figure 32E is a fluorescence micrograph of deep cerebral cortex stained using an anti-KCC3 antibody. The presence of KCC3 is observed as regions of light gray signal. Numerous cortical cell bodies are labeled, as well as cell bodies and processes of pyramidal neurons (arrowheads). Scale bar = 50 μm.

Figure 32F is a fluorescence micrograph of large cell bodies in the superficial cortex stained using an anti-KCC3 antibody. The presence of KCC3 is observed as regions of light gray signal. KCC3 is detected in molecular layers (II) and (III), but is scarce in molecular layer (I). Arrow, edge of brain. Scale bar = 50 μm.

Figure 33A is a schematic depicting a targeting strategy for the generation of a mouse strain genetically deficient in KCC2. Homozygous KCC2- mice were shown to suffer from early neonatal mortality. The targeting strategy shows the structure of the 5' end of Slc12a5/KCC2 gene, position of the 5' probe and structure of the DNA fragment inserted into the gene. The construct was created using pPNT, a vector containing both neomycin and thymidine kinase genes under phosphoglycerate kinase-1 (PGK-1) promoter.

A 2.1-kb Sph I-Xho I fragment (left arm) was ligated upstream of the neomycin cassette, and a 7-kb Bam HI fragment (right arm) was inserted between neomycin and thymidine kinase.

Figure 33B is an autoradiograph depicting Southern-blot analysis of embryonic stem (ES) cell genomic DNA digested with Nhe I and BamH I; the 3-kb band represents the control gene and the 4.5-kb band originates from the mutant gene.

Figure 33C is a photograph depicting PCR analysis of genomic DNA confirms the presence of the mutant gene in two ES cell mutants (1F12 & 2B9). Position of the PCR product is indicated (arrowhead). The upper band represents the primers.

Figure 33D is a graph that charts the life expectancy of wild type mice, heterozygote $KCC2^{-/+}$ mice, and homozygous $KCC2^{-/-}$ mice. Following birth, pups were examined and counted twice a day. Dead pups were removed and genotyped by PCR. Note the striking death rate of homozygote pups between day 11 and 17; all homozygote mutants died by day 17.

Figure 33E is a set of photographs of seizing $KCC2^{-/-}$ mice. Homozygote mutant and control mice were placed upside-down. While the control mouse (bottom right) turned immediately back right side up, the homozygote mutants started to seize. The general stiffness of their limbs can also been seen.

Figure 34A is a graph depicting increased susceptibility to seizure activity in $KCC2^{+/-}$ mice (●) when compared to wild type mice (■). The number of mice showing seizure activity, assayed as described in Example 24, is plotted as a function of the number of injections received (or the number of days injections administered). In this assay, $KCC2^{+/-}$ mice (●) are about 2-fold more susceptible to seizure activity than wild type mice (■).

Figure 34B is a bar graph depicting the percentage of deaths observed in seizure-induced $KCC2^{+/-}$ heterozygous mice (gray bar) and wild type mice (black bar). Approximately 35% of $KCC2^{+/-}$ heterozygous mice die in response to PTZ injection, whereas <5% of wild type animals die following a same PTZ injection.

Figure 35 is a schematic of a targeting construct for the generation of a KCC3 knockout mouse strain. Using PCR, exon 3 was removed and an Xho I site was introduced within the 1450 base pair Sph I fragment. The IRES/bgeo fragment was inserted in the gene at the Xho I site. This construct deletes exon 3, which is not alternatively spliced and is utilized by both KCC3a and KCC3b transcripts.

Figure 36A is a graph depicting the performance of wild type mice (black bar), $KCC3^{+/-}$ heterozygous mice (solid gray bar), and $KCC3^{-/-}$ mutant mice (hatched gray bar) in the rotorod behavioral task. Animals of each genotype were placed on a rotorod, and the time elapsed (Rotorod Latency to Fall in seconds) until the animal fell from the rotorod was determined. All animals were tested at 1 month and 4 months of age, as indicated. A schematic drawing showing the initial position of a mouse on a rotorod is pictured adjacent to the graph.

Figure 36B is a graph depicting the performance of wild type mice (black bar), $KCC3^{+/-}$ heterozygous mice (solid gray bar), and $KCC3^{-/-}$ mutant mice (hatched gray bar) in the wire hang behavioral task. Animals of each genotype were presented with a wire, and the perioe of time (Wire Hang Latency in seconds) during which the mouse gripped the wire was determined. All animals were tested at 1 month and 4 months of age, as indicated. A schematic drawing showing a mouse gripping a wire is pictured adjacent to the graph.

Figure 36C is a graph depicting the performance of wild type mice (black bar), $KCC3^{+/-}$ heterozygous mice (solid gray bar), and $KCC3^{-/-}$ mutant mice (hatched gray bar) in the beam behavioral task. Animals of each genotype were placed on a narrow beam, and the time elapsed (Beam Task Latency in seconds) until the animal fell from the beam was determined during a 60-second interval. All animals were tested at 1 month and 4 months of age, as indicated. A schematic drawing showing the initial position of a mouse on a narrow beam is pictured adjacent to the graph.

Figure 37A is a graph depicting reduced exploratory behavior in $KCC3^{+/-}$ heterozygous mice (pentagon symbol) and $KCC3^{-/-}$ homozygous mice (■) when compared to wild type mice (●). When placed in an activity chamber, the

distance traveled by each mouse was measured in 4 consecutive 5-minute intervals.

Figure 37B is a graph depicting reduced rearing behavior in $KCC3^{+/-}$ heterozygous mice (pentagon symbol) and $KCC3^{-/-}$ homozygous mice (■) when compared to wild type mice (●). When placed in an activity chamber, the number of times each mouse displayed rearing behavior was measured in 4 consecutive 5-minute intervals.

Figure 38 is a graph depicting reduced prepulse inhibition in $KCC2^{+/-}$ heterozygous mice (pentagon symbol) and $KCC3^{-/-}$ homozygous mice (■) when compared to wild type mice (●). The percentage of animals displaying prepulse inhibition is significantly reduced in $KCC3^{-/-}$ mutant animals, and moderately reduced in $KCC3^{+/-}$ heterozygous animals. At the lower right hand corner of the graph, a schematic line drawing shows the magnitude of the acoustic prepulse and pulse stimuli, described further in Example 25.

Figures 39A-39B are micrographs showing representative cross-sections of the sciatic nerve in wild type mice. The myelin coating is thick around large axons (thin arrows). Schwann cells are visible surrounding axons (arrowheads).

Figures 39C-39D are micrographs showing representative cross-sections of the sciatic nerve in $KCC3^{-/-}$ mutant mice. Numerous large axons have a relatively thin myelin coating (thin arrows) compared the degree of myelination in wild type mice. Many axons have a ring-like appearance (thick arrows), although this appearance can also be observed in axons isolated from wild type animals. Degenerating fibers and myelin deposits are also observed (arrowheads).

Figure 40 is a map of the genomic region encoding hKCC2. The top line depicts organization of the KCC2 gene. Exons are represented by boxes, and a subset of the exons are numbered. The line connecting exons represents intronic regions. An enlarged view of hKCC2 exon 1 and the initial segment of intron 1 is presented below the KC2 gene map. A NSRE sequence is conserved in hKCC2, and is located in intron 1 just upstream of a $(GC)_n(CG)_n(GT)_n$ dinucleotide repeat.

Figure 41 summarizes the distribution of KCC2 alleles that display the indicated polymorphic sequence in the region of the $(GC)_n(CG)_n(GT)_n$ dinucleotide repeat (SEQ ID NOs:118-129). Alleles "A" and "B" arbitrarily refer to a first and second allele observed in any one of samples 1-7. Total, the number of individuals carrying each allele.

Summary of Sequences in the Sequence Listing

Odd-numbered SEQ ID NOs:1-15 are nucleotide sequences encoding human, mouse and *xenopus* KCC polypeptides as described in Table 1.

Even-numbered SEQ ID NOs:2-16 and are human, mouse and *xenopus* KCC polypeptide sequences encoded by the immediately preceding nucleotide sequence, *e.g.*, SEQ ID NO:2 is the protein encoded by the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:4 is the protein encoded by the nucleotide sequence of SEQ ID NO:3, *etc.*

SEQ ID NOs:17-19 are mouse and human KCC3 promoter regions.

Even-numbered SEQ ID NOs:20-42 are nucleotide sequences encoding human KCC2 exons 1-24.

SEQ ID NOs:44-110 are genomic sequences encoding human KCC genes.

SEQ ID NO:111 is the nucleotide sequence of human chromosome 5 genomic clone pMS621.

SEQ ID NOs:112 and 113 are the nucleotide and polypeptide sequences, respectively, of *Xenopus* KCC.

SEQ ID NOs:114-115 are KCC2 primer sequences.

SEQ ID NO:116 is the peptide sequence of the KCC3 antigen.

SEQ ID NO:117 is the peptide sequence of the KCC4 antigen.

SEQ ID NOs:118-129 are KCC2 polymorphisms depicted in Figure 44.

SEQ ID NO:130 is the NRSE oligonucleotide referred to for Figures 4A and 4B.

SEQ ID NO:131 is a mouse KCC2 promoter sequence.

Table 1. Summary of Sequences in the Sequence Listing

SEQ ID NO.	description
1-2	human KCC4
3-4	human KCC3a lacking exon 2
5-6	mouse KCC3a lacking exon 2
7-8	mouse KCC3a
9-10	mouse KCC3b
11-12	human KCC2
13-14	mouse KCC4
15-16	human KCC3a
17	human KCC3a promoter
18	mouse KCC3a promoter
19	mouse KCC3b promoter
20-43	coding seqeunces for human KCC2 exons 1-24
44-49	genomic sequences for human KCC2 exons 2-7
50-55	genomic sequences for human KCC2 exons 9-14
56-63	genomic sequences for human KCC2 exons 17-24
64	genomic sequence for human KCC4 exon 2
65	genomic sequence for human KCC4 exon 3
66	genomic sequence for human KCC4 exon 5
67-71	genomic sequences for human KCC4 exons 6-10
72-79	genomic sequences for human KCC4 exons 12-19
80-83	genomic sequences for human KCC4 exons 21-24
84	genomic sequence for human KCC3a exon 1
85	genomic sequence for human KCC3b exon 1
86	genomic sequence for human KCC3 exon 2
87	genomic sequence for human KCC3 exon 3
88	genomic sequence for human KCC3b exon 4
89	genomic sequence for human KCC3a exon 4
90-95	genomic sequences for human KCC3 exons 6-10
96	genomic sequence for human KCC3b exon 12
97	genomic sequence for human KCC3a exon 12
98-110	genomic sequences for human KCC3 exons 13-25
111	human chromosome 5 genomic clone pMS621
112-113	*Xenopus* KCC
114-155	primers to detect KCC2 polymorphism
116	KCC3 peptide antigen
117	KCC4 peptide antigen

Table 1 cont. Summary of Sequences in the Sequence Listing

SEQ ID NO.	description
117	KCC4 peptide antigen
118	sample 1, KCC2 allele A
119	sample 1, KCC2 allele B
120	sample 2, KCC2 allele A
121	sample 3, KCC2 allele A
122	sample 3, KCC2 allele B
123	sample 4, KCC2 allele A
124	sample 4, KCC2 allele B
125	sample 5, KCC2 allele A
126	sample 5, KCC2 allele B
127	sample 6, KCC2 allele A
128	sample 6, KCC2 allele B
129	sample 7, KCC2 allele A
130	mKCC2 NSRE oligonucleotide
131	mKCC2 promoter sequence

Detailed Description of the Invention

The present invention teaches novel members of the KCC gene family and provides a novel human KCC2 (representative embodiments set forth in SEQ ID NOs:11-12), a novel, alternatively-spliced form of the human KCC3 gene (referred to herein as "hKCC3a", representative embodiments set forth in SEQ ID NOs:15-16) and the novel isoforms, mouse KCC3a (representative embodiments set forth in SEQ ID NOs:7-8) and mouse KCC3b (representative embodiments set forth in SEQ ID NOs:9-10), human and mouse KCC3a lacking exon 2 (mKCC3a-2m and hKCC3a-2m, representative embodiments set forth in SEQ ID NOs:3-6), human KCC4 (representative embodiments set forth in SEQ ID NOs:1-2) and mouse KCC4 (representative embodiments set forth in SEQ ID NOs:13-14), and a *Xenopus* KCC gene (representative embodiments set forth as SEQ ID NOs:112-113).

The four predicted KCC proteins share 65-75% identity in primary structure. The KCC proteins also share a common predicted membrane topology with hydrophilic amino- and carboxy-terminal cytoplasmic domains flanking a central hydrophobic core of 12 highly conserved transmembrane

(TM) segments. A large glycosylated extracellular loop is predicted between TM5 and TM6. The predicted cytoplasmic domains, in particular the carboxy-terminal (C-terminal) domain, contain segments of substantial homology interspersed by variable segments. The extreme C-terminus is completely identical in the four proteins, suggesting a crucial functional role. No one predicted phosphorylation site is conserved in the four KCCs, suggesting significant variation in post-transcriptional regulation. Given the known role of protein phosphorylation/dephosphorylation in cell volume regulation (Hoffmann & Dunham (1995) *Int Rev Cytol* 161:173-262), the volume-sensitivity of the four KCCs differs substantially. Although the TM segments are in general highly conserved, there is intriguing variation within TM2, a TM segment thought to influence cation affinity in the Na^+-K^+-$2Cl^-$ cotransporters. TM4 and TM7, which have been implicated in anion affinity in the Na^+-K^+-$2Cl^-$ cotransporters (Isenring et al. (1998) *J Gen Physiol* 112(5):549-558). The kinetic characteristics of the four KCCs are heterogeneous.

The four KCCs differ in tissue distribution. KCC1 is almost ubiquitous, consistent with a "house-keeping" role in cell volume regulation. KCC2 is restricted to neuronal cells in the nervous system and retina, but can be induced *in vitro* by the differentiation of NT2 teratocarcinoma cells into neurons by retinoic acid (Pleasure & Lee (1993) *J Neurosci Res* 35(6):585-602). KCC4, although widely expressed, is most abundant in heart and kidney, and KCC3 is expressed in muscle, brain, lung, heart, and kidney. As shown in Figures 29A-29K, KCC3b predominates in kidney, whereas KCC3a predominates in brain.

Thus, the present invention pertains to isolated and purified nucleic acids encoding potassium-chloride cotransporter polypeptides, to isolated and purified potassium-chloride cotransporter polypeptides, to the characterization of the role played by the potassium-chloride cotransporter in modulating potassium and chloride levels within and outside cells, and to the characterization of downstream processes affected by such modulation.

Potassium-chloride cotransporters play important physiological roles in multiple tissues and processes, including salt transport in epithelial tissues and kidney, potassium secretion in the kidney, cell volume regulation, and the

regulation of cellular chloride concentration and extracellular potassium concentration. Hence, potassium-chloride cotransporters are of major pharmacological interest, particularly for disorders such as hypertension, epilepsy, and sickle-cell anemia.

The four human KCC genes are localized on different chromosomes, as described in Examples. KCC1 is on chromosome 16q22.1, KCC2 is on 20q13, KCC3 on 15q14, and KCC4 on 5p15. KCC3 is a functional and positional candidate for a number of monogenic and polygenic diseases. The gene is located within the critical region for Andermann's syndrome (Casaubon et al. (1996) *Am J Hum Genet* 58(1):28-34). This recessive disorder comprises agenesis of the corpus callosum, a progressive peripheral neuropathy, developmental delay, and mental retardation. There is some phenotypic overlap with recessive familial spastic paraparesis, a milder disorder that also maps to 15q14 and might be allelic to Andermann's syndrome (Martinez Murillo et al. (1999) *Neurology* 53(1):50-56).

In addition to these monogenic disorders, markers flanking KCC3 are linked to juvenile myoclonic epilepsy and familial rolandic epilepsy, two subtypes of idiopathic epilepsy with a strong genetic component (Neubauer et al. (1998) *Neurology* 51(6):1608-1612; Elmslie et al. (1997) *Hum Mol Genet* 6(8):1329-1334). Patients with juvenile myoclonic epilepsy, a common subtype of idiopathic generalized epilepsy (4-10% of all epilepsy) (Callenbach & Brouwer (1997) *Clin Neurol Neurosurg* 99(3):159-171), suffer from both myoclonic and generalized tonic-clonic seizures. Many have a family history of other subtypes of epilepsy, in addition to abnormal EEGs in up to 10% of healthy family members.

Loss of heterozygosity for the genetic markers near the hKCC3 gene has been reported in metastatic brain and breast tumors (Wick et al. (1996) *Oncogene* 12(5):973-978), suggesting that this segment of chromosome 15q14 harbors a tumor suppressor. Markers near KCC3 have also been linked to inherited susceptibility to colonic malignancy (Tomlinson et al. (1999) *Gastroenterology* 116(4):789-795).

Based on its localization in renal thick ascending limb, in addition to functional evidence for K^+-Cl^- cotransport in this nephron segment (Amlal et al. (1994) *American Journal of Physiology* 267(6 Pt 1):C1607-1615), hKCC4 is a gene candidate for the subtypes of Bartter's syndrome not due to mutations in NKCC2 (Simon et al. (1996) *Nat Genet* 13(2):183-188), ROMK (Simon et al. (1996) *Nat Genet* 14:152-156), or CLC-NKB (Simon et al. (1997) *Nat Genet* 17:171-178). Linkage analysis implicates the genomic region containing human KCC4 in variation in bitter taste perception (Reed et al. (1999) *Am J Hum Genet* 64(5):1478-1480). Based on synteny to human chromosome 5p15, mouse KCC4 is located on chromosome 13, in a region linked to audiogenic seizures in the "frings" mouse strain (Skradski et al. (1998) *Genomics* 49(2):188-192).

The functional and physiological data for the KCCs also implicates these transporters in human disease. Thus red cell K^+-Cl^- cotransport is implicated in the dehydration and subsequent sickling of red cells in sickle cell anemia (Brugnara et al. (1986) *Science* 232(4748):388-390; De Franceschi et al. (1996) *Blood* 88(7):2738-2744). The expression of KCC3 and KCC4 in renal tubular cells, including proximal tubule, implicates K^+-Cl^- cotransport in renal salt handling and hence in hypertension. In addition, KCC4 is expressed in macula densa cells, where its activity can modulate glomerular filtration and renin release.

Finally, the observation that mice genetically deficient in KCC2 have a seizure disorder (Figure 36) implicates this transporter in epileptogenesis. The magnitude of the electrochemical gradient for Cl^- modulates the response of neurons to stimuli that affect chloride conductance, such as the neurotransmitter γ-aminobutyric acid (GABA) (Misgeld et al. (1986) *Science* 232(4756):1413-1415; Miles (1999) *Nature* 397(6716):215-216). In neurons with robust inward transport of Cl^-, intracellular Cl^- concentration (Cl^-_i) is high and $GABA_A$ receptor stimulation is depolarizing and excitatory. In contrast, in cells with outward Cl^- transport $GABA_A$ activation is hyperpolarizing and inhibitory (Miles (1999) *Nature* 397(6716):215-216).

The inward and outward transport of Cl^- in neurons are encoded by the Na^+-K^+-$2Cl^-$ cotransporter NKCC1 and the KCCs, respectively. See Misgeld et

al. (1986) *Science* 232(4756):1413-1415; Clayton et al. (1998) *Brain Res Dev Brain Res* 109(2):281-292. NKCC1 in the central nervous system is developmentally regulated, with high levels of expression at birth and the first few postnatal days, and decreased expression thereafter (Hubner et al. (2001) *Mech Dev* 102:267-269). In contrast, the expression of KCC2 increases dramatically during postnatal development (Lu et al. (1999) *J Neurobiology* 39:558-568; Rivera et al. (1999) *Nature* 397:251-255; Clayton et al. (1998) *Brain Res Dev Brain Res* 109(2):281-292). These developmental events correlate with a switch of the $GABA_A$ effect, from depolarizing to hyperpolarizing, during the first weeks of postnatal life.

Although other pathways can also function in neuronal Cl^{-i} homeostasis (Clayton et al. (1998) *Brain Res Dev Brain Res* 109(2):281-292), recent anti-sense experiments in cultured neurons strongly implicate KCC2 (Rivera et al. (1999) *Nature* 397:251-255), as does the phenotype of the KCC2 knockout mouse. The resulting change in the neuronal response to $GABA_A$ and other neurotransmitters is thought to have important effects on both neuronal development and neuronal remodeling (Miles (1999) *Nature* 397(6716): 215-216). Trauma to cultured neurons also has effects on neuronal Cl^{-i} and response to GABA (van den Pol et al. (1996) *J Neurosci* 16(13):4283-4292). The pivotal role of KCC2 and other K^+- Cl^- transporters in regulating neuronal K^+ and Cl^- strongly implicates these transporters in the generation of seizure activity and epilepsy (Payne (1997) *Am J Physiol* 273:C1516-C1525). However, the KCCs can also affect epileptiform activity via effects on cell volume (Hochman et al. (1995) *Science* 270(5233):99-102).

Potassium-chloride cotransport is also envisioned to play a role in the generation of epileptiform activity in epilepsy, and in red-cell dehydration in sickle-cell anemia. The evident importance of potassium-chloride cotransport in salt absorption by the kidney also implicates potassium-chloride cotransporters in salt balance and hypertension. Immunofluorescence with isoform-specific antibodies localizes KCC3 and KCC4 to the basolateral membranes of mouse renal proximal tubule. Swelling-activated K^+-Cl^- cotransport in this and other nephron segments is postulated to play a major role in transepithelial salt

transport along the nephron (Mount et al. (1999) *J Biol Chem* 274(23):16355-16362). Moreover, the localization of KCC4 at the basolateral membrane of macula densa cells implicates this transporter in both tubuloglomerular feedback and tubular regulation of renin release, since both processes involve sensing and transport of chloride by this highly specialized nephron segment (Mount et al. (1997) *J Membr Biol* 158:177-186).

There are thus a large number of applications for drugs that target potassium-chloride cotransporters. The paucity of molecular information has hampered progress in determining the physiological and pathophysiological significance of potassium-chloride cotransport. The cloning of a human KCC2, and of KCC3 and KCC4 from various mammals is thus a major advance in this field. The development of isoform-specific antibodies to study the tissue localization of potassium-chloride cotransporters is facilitated, and is crucial for gene disruption studies of the four potassium-chloride cotransporter isoforms. The functional comparison of the four KCCs also impacts the study of their regulation and role. For example, kinetic comparisons yield important structure-function data, including ion-binding sites. Pharmacological comparison also provide for the development of isoform- and class-specific inhibitors and activators.

Summarily, the identification of the genes that encode KCC2 in humans, KCC3 in mice and humans and KCC4 in mice and humans, the cloning of the cDNAs and the expression of the proteins affords the molecular tools required for modulating potassium-chloride homeostasis, and has application in the development of pharmacological and/or therapeutic treatments for various disorders.

A. Polypeptides and Nucleic Acids

As used in the following detailed description and in the claims, the terms "KCC", "KCC2", "KCC3" and "KCC4" include nucleic acids and polypeptides encoding potassium-chloride cotransporters. The terms "KCC", "KCC2", "KCC3" and "KCC4" include invertebrate homologs; preferably, KCC, KCC2, KCC3 and KCC4 nucleic acids and polypeptides are isolated from eukaryotic

sources. "KCC", "KCC2", "KCC3" and "KCC4" further include vertebrate homologs of potassium-chloride cotransporter family members, including, but not limited to, mammalian and avian homologs. Representative mammalian homologs of potassium-chloride cotransporter gene family members include, but are not limited to, murine and human homologs. The term "KCC", as used herein, can generally refer to any of the disclosed KCC nucleic acids and polypeptides, e.g. KCC2, KCC3, KCC4 or *Xenopus* KCC (xKCC).

The terms "KCC gene product", "KCC2 gene product","KCC3 gene product", "KCC4 gene product", "KCC protein", "KCC2 protein", "KCC3 protein", "KCC4 protein", "KCC polypeptide", "KCC2 polypeptide", "KCC3 polypeptide" and "KCC4 polypeptide" refer to peptides having amino acid sequences which are substantially identical to native amino acid sequences from the organism of interest and which are biologically active in that they comprise all or a part of the amino acid sequence of a potassium-chloride cotransporter, or cross-react with antibodies raised against a KCC, KCC2, KCC3 or KCC4 polypeptide, or retain all or some of the biological activity of the native amino acid sequence or protein. Such biological activity can include immunogenicity.

The terms "KCC gene product", "KCC2 gene product","KCC3 gene product", "KCC4 gene product", "KCC protein", "KCC2 protein", "KCC3 protein", "KCC4 protein", "KCC polypeptide", "KCC2 polypeptide", "KCC3 polypeptide" and "KCC4 polypeptide" also include analogs of potassium-chloride cotransporter molecules. By "analog" is intended that a DNA or peptide sequence can contain alterations relative to the sequences disclosed herein, yet retain all or some of the biological activity of those sequences. Analogs can be derived from genomic nucleotide sequences as are disclosed herein or from other organisms, or can be created synthetically. Those skilled in the art will appreciate that other analogs, as yet undisclosed or undiscovered, can be used to design and/or construct potassium-chloride cotransporter analogs. There is no need for a "KCC gene product", "KCC2 gene product","KCC3 gene product", "KCC4 gene product", "KCC protein", "KCC2 protein", "KCC3 protein", "KCC4 protein", "KCC polypeptide", "KCC2 polypeptide", "KCC3 polypeptide" and "KCC4 polypeptide" to comprise all or substantially all of the amino acid

sequence of a native potassium-chloride cotransporter gene product. Shorter or longer sequences are anticipated to be of use in the invention; shorter sequences are herein referred to as "segments." Thus, the terms "KCC gene product", "KCC2 gene product","KCC3 gene product", "KCC4 gene product", "KCC protein", "KCC2 protein", "KCC3 protein", "KCC4 protein", "KCC polypeptide", "KCC2 polypeptide", "KCC3 polypeptide" and "KCC4 polypeptide" also include fusion or recombinant potassium-chloride cotransporter polypeptides and proteins comprising sequences of the present invention. Methods of preparing such proteins are known in the art.

The terms "KCC gene", "KCC2 gene", "KCC3 gene", "KCC4 gene", "KCC gene sequence", "KCC2 gene sequence", "KCC3 gene sequence", "KCC4 gene sequence", "KCC gene segment", "KCC2 gene segment", "KCC3 gene segment", and "KCC4 gene segment" refer to any DNA sequence that is substantially identical to a polynucleotide sequence encoding a potassium-chloride cotransporter gene product, protein or polypeptide as defined above, and can also comprise any combination of associated control sequences. The terms also refer to RNA, or antisense sequences, complementary to such DNA sequences. As used herein, the term "DNA segment" refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Furthermore, a DNA segment encoding a potassium-chloride cotransporter polypeptide refers to a DNA segment that contains KCC2, KCC3 or KCC4 coding sequences, yet is isolated away from, or purified free from, total genomic DNA of a source species, such as *Homo sapiens*. Included within the term "DNA segment" are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phages, viruses, and the like.

The term "substantially identical", when used to define either a KCC, KCC2, KCC3 or KCC4 gene product or amino acid sequence, or a KCC, KCC2, KCC3 or KCC4 gene or nucleic acid sequence, means that a particular sequence varies from the sequence of a natural KCC, KCC2, KCC3 or KCC4 by one or more deletions, substitutions, or additions, the net effect of which is to retain at least some of biological activity of the natural gene, gene product, or

sequence. Such sequences include "mutant" sequences, or sequences in which the biological activity is altered to some degree but retains at least some of the original biological activity.

Alternatively, DNA analog sequences are "substantially identical" to specific DNA sequences disclosed herein if: (a) the DNA analog sequence is derived from coding regions of the natural KCC, KCC2, KCC3 or KCC4 gene; or (b) the DNA analog sequence is capable of hybridization of DNA sequences of (a) under stringent conditions and which encode biologically active KCC, KCC2, KCC3 or KCC4 gene product; or (c) the DNA sequences are degenerate as a result of alternative genetic code to the DNA analog sequences defined in (a) and/or (b). Substantially identical analog proteins will be greater than about 60% identical to the corresponding sequence of the native protein. Sequences having lesser degrees of identity but comparable biological activity are considered to be equivalents. In determining nucleic acid sequences, all subject nucleic acid sequences capable of encoding substantially similar amino acid sequences are considered to be substantially similar to a reference nucleic acid sequence, regardless of differences in codon sequences or substitution of equivalent amino acids to create biologically functional equivalents.

Sequence identity or percent similarity of a DNA or peptide sequence can be determined, for example, by comparing sequence information using the GAP computer program, available from the University of Wisconsin Geneticist Computer Group. The GAP program utilizes the alignment method of Needleman et al. (1970) *J Mol Biol* 48:443, as revised by Smith et al. (1981) *Adv Appl Math* 2:482. Briefly, the GAP program defines similarity as the number of aligned symbols (*i.e.,* nucleotides or amino acids) that are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred parameters for the GAP program are the default parameters, which do not impose a penalty for end gaps. See Schwartz et al. (1979) *Nuc Acids Res* 6(2):745-755; Gribskov et al. (1986) *Nuc Acids Res* 14(1):327-334.

In certain embodiments, the invention concerns the use of KCC, KCC2, KCC3 and KCC4 genes and gene products that include within their respective sequences a sequence which is essentially that of a KCC, KCC2, KCC3 or

KCC4 gene, or the corresponding protein. The term "a sequence essentially as that of a KCC, KCC2, KCC3 or KCC4 gene", means that the sequence is substantially identical or substantially similar to a portion of a KCC, KCC2, KCC3 or KCC4 gene and contain a minority of bases or amino acids (whether DNA or protein) which are not identical to those of a KCC, KCC2, KCC3 or KCC4 protein or a KCC, KCC2, KCC3 or KCC4 gene, or which are not a biologically functional equivalent. The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein. Nucleotide sequences are "essentially the same" where they have between about 70% and about 80% or more preferably, between about 81% and about 90%, or even more preferably, between about 91% and about 99%; of nucleic acid residues which are identical to the nucleotide sequence of a KCC, KCC2, KCC3 or KCC4 gene. Similarly, peptide sequences which have about 35%, or 45%, or preferably from 45-55%, or more preferably 55-65%, or most preferably 65% or greater amino acids which are identical or functionally equivalent or biologically functionally equivalent to the amino acids of a KCC, KCC2, KCC3 or KCC4 polypeptide will be sequences which are "essentially the same".

KCC, KCC2, KCC3 or KCC4 gene products and KCC, KCC2-, KCC3- or KCC4- encoding nucleic acid sequences, which have functionally equivalent codons, are also covered by the invention. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the ACG and AGU codons for serine. Thus, when referring to the sequence examples presented in SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and 112 applicants contemplate substitution of functionally equivalent codons of Table 2 into the sequence examples of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and 112. Thus, applicants are in possession of amino acid and nucleic acids sequences which include such substitutions but which are not set forth herein in their entirety for convenience.

TABLE 2 - Functionally Equivalent Codons

Amino Acids			Codons
Alanine	Ala	A	GCA GCC GCG GCU
Cysteine	Cys	C	UGC UGU

Aspartic Acid	Asp	D	GAC GAU
Glumatic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	UUC UUU
Glycine	Gly	G	GGA GGC GGG GGU
Histidine	His	H	CAC CAU
Isoleucine	Ile	I	AUA AUC AUU
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	UUA UUG CUA CUC CUG CUU
Methionine	Met	M	AUG
Asparagine	Asn	N	AAC AAU
Proline	Pro	P	CCA CCC CCG CCU
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGU
Serine	Ser	S	ACG AGU UCA UCC UCG UCU
Threonine	Thr	T	ACA ACC ACG ACU
Valine	Val	V	GUA GUC GUG GUU
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAC UAU

It will also be understood by those of skill in the art that amino acid and nucleic acid sequences can include additional residues, such as additional NB or C-terminal amino acids or 5' or 3' nucleic acid sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence retains biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences which can, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or can include various internal sequences, *i.e.*, introns, which are known to occur within genes.

The present invention also encompasses the use of nucleotide segments that are complementary to the sequences of the present invention. Nucleic acid sequences which are "complementary" are those, which are base-paired according to the standard Watson-Crick complementarity rules. As used

herein, the term "complementary sequences" means nucleic acid sequences which are substantially complementary, as can be assessed by the same nucleotide comparison set forth above, or is defined as being capable of hybridizing to the nucleic acid segment in question under relatively stringent conditions such as those described herein. A particular example of a contemplated complementary nucleic acid segment is an antisense oligonucleotide.

One technique in the art for assessing complementary sequences and/or isolating complementary nucleotide sequences is hybridization. Nucleic acid hybridization will be affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of nucleotide base mismatches between the hybridizing nucleic acids, as will be readily appreciated by those skilled in the art. Stringent temperature conditions will generally include temperatures in excess of about 30°C, typically in excess of about 37°C, and preferably in excess of about 45°C. Stringent salt conditions will ordinarily be less than about 1,000 mM, typically less than about 500 mM, and preferably less than about 200 mM. However, the combination of parameters is much more important than the measure of any single parameter. See *e.g.*, Wethmur & Davidson (1968) *J Mol Biol* 31:349-370. Determining appropriate hybridization conditions to identify and/or isolate sequences containing high levels of homology is well known in the art. See *e.g.,* Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

For the purposes of specifying conditions of high stringency, preferred conditions are salt concentration of about 200 mM and temperature of about 45°C. One example of such stringent conditions is hybridization at 4XSSC, at 65°C, followed by a washing in 0.1XSSC at 65°C for one hour. Another exemplary stringent hybridization scheme uses 50% formamide, 4XSSC at 42°C. As used herein, "stringent conditions" means conditions of high stringency, for example 6XSSC, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin, 0.1% sodium dodecyl sulfate, 100 µg/ml salmon sperm

DNA and 15% formamide at 68°C. Nucleic acids having sequence similarity are detected by hybridization under low stringency conditions, for example, at 50°C and 10XSSC (0.9 M NaCl/0.09 M sodium citrate) and remain bound when subjected to washing at 55°C in 1XSSC. Sequence identity can be determined by hybridization under stringent conditions, for example, at 50°C or higher and 0.1XSSC (9 mM NaCl/0.9 mM sodium citrate).

Nucleic acids that are substantially identical to the provided KCC sequences, *e.g.,* allelic variants, genetically altered versions of the gene, *etc.*, bind to the provided KCC sequences under stringent hybridization conditions. By using probes, particularly labeled probes of DNA sequences, one can isolate homologous or related genes. The source of homologous genes can be any species, *e.g.,* primate species, particularly human; rodents, such as rats and mice, canines, felines, bovines, ovines, equines, yeast, nematodes, *etc.*

Between mammalian species, *e.g.,* human, mouse and *xenopus*, homologs have substantial sequence similarity, *i.e.* at least 75% sequence identity between nucleotide sequences. Sequence similarity is calculated based on a reference sequence, which can be a subset of a larger sequence, such as a conserved motif, coding region, flanking region, *etc.* A reference sequence will usually be at least about 18 nucleotides long, more usually at least about 30 nucleotides long, and can extend to the complete sequence that is being compared. Algorithms for sequence analysis are known in the art, such as BLAST, described in Altschul et al. (1990) *J Mol Biol* 215:403-410. The sequences provided herein are essential for recognizing KCC related and homologous proteins in database searches.

At a biological level, identity is just that, *i.e.* the same amino acid at the same relative position in a given family member of a gene family. Homology and similarity are generally viewed as broader terms. For example, biochemically similar amino acids, for example leucine and isoleucine or glutamate/aspartate, can be present at the same position - these are not identical per se, but are biochemically "similar". As disclosed herein, these are referred to as conservative differences or conservative substitutions. This differs from a conservative mutation at the DNA level, which changes the

nucleotide sequence without making a change in the encoded amino acid, *e.g.*, TCC to TCA, both of which encode serine.

The KCCs disclosed herein are thus homologous proteins, but when percentages are referred to herein, it is meant to refer to percent identity. The percent identities referenced herein were generated by alignments with the program GeneWorks (Oxford Molecular, Inc. of Campbell, California) and/or the BLAST program at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Another commonly used alignment program is entitled CLUSTAL W and is described in Thompson et al. (1994) *Nucleic Acids Res* 22(22):4673-4680, among other places.

Probe sequences can also hybridize specifically to duplex DNA under certain conditions to form triplex or other higher order DNA complexes. The preparation of such probes and suitable hybridization conditions are disclosed herein and are known in the art.

The term "gene" is used for simplicity to refer to a functional protein, polypeptide or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences and cDNA sequences. Preferred embodiments of genomic and cDNA sequences are disclosed herein.

In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences, which encode a potassium-chloride cotransporter polypeptide that includes within its amino acid sequence an amino acid sequence of the present invention. In other particular embodiments, the invention concerns recombinant vectors incorporating DNA segments, which encode a protein comprising the amino acid sequence of a human potassium-chloride cotransporter.

A.1. Biologically Functional Equivalents

As mentioned above, modifications and changes can be made in the structure of the potassium-chloride cotransporter proteins and peptides described herein and still constitute a molecule having like or otherwise desirable characteristics. For example, certain amino acids can be substituted for other amino acids in a protein structure without appreciable loss of interactive capacity with, for example, structures in the nucleus of a cell. Since

it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence (or the nucleic acid sequence encoding it) to obtain a protein with the same, enhanced, or antagonistic properties. Such properties can be achieved by interaction with the normal targets of the native protein, but this need not be the case, and the biological activity of the invention is not limited to a particular mechanism of action. It is thus contemplated in accordance with the present invention that various changes can be made in the sequence of the potassium-chloride cotransporter proteins and peptides or underlying nucleic acid sequence without appreciable loss of their biological utility or activity.

Biologically functional equivalent peptides, as used herein, are peptides in which certain, but not most or all, of the amino acids can be substituted. Thus, when referring to the sequence examples presented in SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and/or 112, applicants contemplate substitution of codons that encode biologically equivalent amino acids as described herein into the sequence examples of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and/or 112. Thus, applicants are in possession of amino acid and nucleic acids sequences which include such substitutions but which are not set forth herein in their entirety for convenience.

Alternatively, functionally equivalent proteins or peptides can be created via the application of recombinant DNA technology, in which changes in the protein structure can be engineered, based on considerations of the properties of the amino acids being exchanged, *e.g.,* substitution of Ile for Leu at amino acids 53, 86 and/or 1028 for hKCC4 in SEQ ID NOs:1-2; substitution of Ile for Leu at amino acids 35, 472 and/or 1075 for hKCC3a2m in SEQ ID NOs:3-4; substitution of Ile for Leu at amino acids 102, 631 and/or 1113 for mKCC3a2m in SEQ ID NOs:5-6; substitution of Ile for Leu at amino acids 35, 308 and/or 848 for mKCC3a in SEQ ID NOs:7-8; substitution of Ile for Leu at amino acids 66, 537 and/or 974 for mKCC3b in SEQ ID NOs:9-10; substitution of Ile for Leu at amino acids 120, 358 and/or 916 for hKCC2 in SEQ ID NOs:11-12; substitution of Ile for Leu at amino acids 71, 467 and/or 639 for mKCC4 in SEQ ID NOs:13-

14; and substitution of Ile for Leu at amino acids 35, 346 and/or 789 for hKCC3a in SEQ ID NOs:15-16. Changes designed by man can be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the antigenicity of the protein or to test KCC mutants in order to examine KCC transport activity, or other activity at the molecular level.

Amino acid substitutions, such as those which might be employed in modifying the potassium-chloride cotransporter proteins and peptides described herein, are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. An analysis of the size, shape and type of the amino acid side-chain substituents reveals that arginine, lysine and histidine are all positively charged residues; that alanine, glycine and serine are all of similar size; and that phenylalanine, tryptophan and tyrosine all have a generally similar shape. Therefore, based upon these considerations, arginine, lysine and histidine; alanine, glycine and serine; and phenylalanine, tryptophan and tyrosine; are defined herein as biologically functional equivalents. Other biologically functionally equivalent changes will be appreciated by those of skill in the art.

In making biologically functional equivalent amino acid substitutions, the hydropathic index of amino acids can be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+ 4.5); valine (+ 4.2); leucine (+ 3.8); phenylalanine (+ 2.8); cysteine (+ 2.5); methionine (+ 1.9); alanine (+ 1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte et al. (1982) *J Mol Biol* 157:105, herein incorporated herein by reference). It is known that certain amino acids can be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological

activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ± 2 of the original value is preferred, those, which are within ± 1 of the original value, are particularly preferred, and those within ± 0.5 of the original value are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent No. 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, *i.e.* with a biological property of the protein. It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent protein.

As detailed in U.S. Patent No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+ 3.0); lysine (+ 3.0); aspartate (+ 3.0 ± 1); glutamate (+ 3.0 ± 1); serine (+ 0.3); asparagine (+ 0.2); glutamine (+ 0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ± 2 of the original value is preferred, those, which are within ± 1 of the original value, are particularly preferred, and those within ± 0.5 of the original value are even more particularly preferred.

While discussion has focused on functionally equivalent polypeptides arising from amino acid changes, it will be appreciated that these changes can be effected by alteration of the encoding DNA, taking into consideration also that the genetic code is degenerate and that two or more codons can code for the same amino acid.

Thus, it will also be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NOs:1-16 and 112-113. Recombinant vectors and isolated DNA segments can therefore

variously include the potassium-chloride cotransporter polypeptide-encoding region itself, include coding regions bearing selected alterations or modifications in the basic coding region, or include larger polypeptides which nevertheless comprise potassium-chloride cotransporter polypeptide-encoding regions or can encode biologically functional equivalent proteins or peptides which have variant amino acid sequences. Biological activity of a potassium chloride cotransporter can be determined, for example, measuring the amount of $^{86}Rb^{+}$ uptake following transformation of the DNA of interest into *Xenopus* laevis oocytes, as disclosed herein.

In particular embodiments, the invention concerns gene therapy methods that use isolated DNA segments and recombinant vectors incorporating DNA sequences which encode a protein comprising an amino acid sequence of any of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16 and 113. In other particular embodiments, the invention concerns isolated DNA sequences and recombinant DNA vectors incorporating DNA sequences which encode a protein comprising the amino acid sequence of the potassium-chloride cotransporter protein from human or mouse tissue. In certain other embodiments, the invention concerns isolated DNA segments and recombinant vectors that comprise a nucleic acid sequence essentially as set forth in any of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15 and/or 112.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, can be combined with other DNA sequences, such as promoters, enhancers, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length can vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length can be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments can be prepared which include a short stretch complementary to a nucleic acid sequence set forth in any of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15 and/or 112, such as about 10 nucleotides, and which are up to 10,000 or 5,000 base pairs in length, with segments of 3,000 being preferred

in certain cases. DNA segments with total lengths of about 4,000, 3,000, 2,000, 1,000, 500, 200, 100, and about 50 base pairs in length are also contemplated to be useful.

The DNA segments of the present invention encompass biologically functional equivalent potassium-chloride cotransporter proteins and peptides. Such sequences can rise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides can be created via the application of recombinant DNA technology, in which changes in the protein structure can be engineered, based on considerations of the properties of the amino acids being exchanged. Changes can be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the antigenicity of the protein or to test potassium-chloride cotransporter mutants in order to examine activity in the modulation of potassium-chloride cotransporter, or other activity at the molecular level. Site-directed mutagenesis techniques are known to those of skill in the art and are disclosed herein.

The invention further encompasses fusion proteins and peptides wherein the potassium-chloride cotransporter coding region is aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes.

Recombinant vectors form important further aspects of the present invention. Particularly useful vectors are those in which the coding portion of the DNA segment is positioned under the control of a promoter. The promoter can be that naturally associated with the potassium-chloride cotransporter gene, as can be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR technology and/or other methods known in the art, in conjunction with the compositions disclosed herein.

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or

heterologous promoter is a promoter that is not normally associated with a potassium-chloride cotransporter gene in its natural environment. Such promoters can include promoters isolated from bacterial, viral, eukaryotic, or mammalian cells. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology (See, *e.g.,* Sambrook et al., eds. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.). The promoters employed can be constitutive or inducible and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. Appropriate promoter systems contemplated for use in high-level expression include, but are not limited to, the vaccinia virus promoter and the baculovirus promoter.

In an alternative embodiment, the present invention provides an expression vector comprising a polynucleotide that encodes a biologically active potassium-chloride cotransporter polypeptide in accordance with the present invention. Also preferably, an expression vector of the present invention comprises a polynucleotide that encodes human or mouse potassium-chloride cotransporter gene product. More preferably, an expression vector of the present invention comprises a polynucleotide that encodes a polypeptide comprising an amino acid residue sequence of any of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, adn 113. More preferably, an expression vector of the present invention comprises a polynucleotide comprising the nucleotide sequence of any of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and 112. Even more preferably, an expression vector of the invention comprises a polynucleotide operatively linked to an enhancer-promoter. More preferably still, an expression vector of the invention comprises a polynucleotide operatively linked to a prokaryotic promoter. Alternatively, an expression vector of the present invention comprises a polynucleotide operatively linked to an enhancer-promoter that is a eukaryotic promoter and the expression vector further comprises a

polyadenylation signal that is positioned 3' of the carboxy-terminal amino acid and within a transcriptional unit of the encoded polypeptide.

In yet another embodiment, the present invention provides a recombinant host cell transfected with a polynucleotide that encodes a biologically active potassium-chloride cotransporter polypeptide in accordance with the present invention. SEQ ID NOs:1-16 and 112-113 set forth nucleotide and amino acid sequences from exemplary vertebrates, human, mouse, and *xenopus*. Also contemplated by the present invention are homologous or biologically functionally equivalent polynucleotides and potassium-chloride cotransporter polypeptides found in other vertebrates, including particularly rat and bovine homologs. Preferably, a recombinant host cell of the present invention is transfected with the polynucleotide that encodes human or mouse potassium-chloride cotransporter polypeptide. More preferably, a recombinant host cell of the present invention is transfected with the polynucleotide sequence encoding or set forth in any of SEQ ID NOs:1-16 and 112-113. Most preferably, a recombinant host cell is a mammalian cell.

In another aspect, a recombinant host cell of the present invention is a prokaryotic host cell, including parasitic and bacterial cells. Preferably, a recombinant host cell of the invention is a bacterial cell, preferably a strain of Escherichia coli. More preferably, a recombinant host cell comprises a polynucleotide under the transcriptional control of regulatory signals functional in the recombinant host cell, wherein the regulatory signals appropriately control expression of the potassium-chloride cotransporter polypeptide in a manner to enable all necessary transcriptional and post-transcriptional modification.

In yet another embodiment, the present invention provides a process of preparing a potassium-chloride cotransporter polypeptide comprising transfecting a cell with polynucleotide that encodes a biologically active potassium-chloride cotransporter polypeptide in accordance with the present invention, to produce a transformed host cell, and maintaining the transformed host cell under biological conditions sufficient for expression of the polypeptide. The polypeptide can be isolated if desired, using any suitable technique. The

host cell can be a prokaryotic or eukaryotic cell. Preferably, the prokaryotic cell is a bacterial cell of *Escherichia coli*. More preferably, a polynucleotide transfected into the transformed cell comprises the nucleotide base sequence of any of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and 112. SEQ ID NOs:1-16 and 112-113 set forth nucleotide and amino acid sequences for representative vertebrates, human, mouse and *Xenopus*. Also contemplated by the present invention are homologs or biologically equivalent potassium-chloride cotransporter polynucleotides and polypeptides found in other vertebrates, particularly warm-blooded vertebrates, more particularly mammals, and even more particularly bovine and rat homologs.

As mentioned above, in connection with expression embodiments to prepare recombinant potassium-chloride cotransporter proteins and peptides, it is contemplated that longer DNA segments will most often be used, with DNA segments encoding the entire potassium-chloride cotransporter protein, functional domains or cleavage products thereof, being most preferred. However, it will be appreciated that the use of shorter DNA segments to direct the expression of potassium-chloride cotransporter peptides or core regions, such as can be used to generate anti-potassium-chloride cotransporter antibodies, also falls within the scope of the invention.

DNA segments which encode peptide antigens from about 15 to about 50 amino acids in length, or more preferably, from about 15 to about 30 amino acids in length are contemplated to be particularly useful. DNA segments encoding peptides will generally have a minimum coding length in the order of about 45 to about 150, or to about 90 nucleotides. DNA segments encoding full length proteins can have a minimum coding length on the order of about 4,000 or 5,000 nucleotides for a protein in accordance with any of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and 112. DNA segments of the present invention can contain 300, 400, 500, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 4,500, or up to 5,000 nucleotides. Peptides of the present invention can contain 10, 20, 50, 100, 200, 300, 400, 500, 750, 1,000, or up to 1,500 amino acids.

A.2. Sequence Modification Techniques

Modifications to the potassium-chloride cotransporter proteins and peptides described herein can be carried out using techniques known in the art, including site directed mutagenesis. Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants; for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 30 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art as exemplified by publications (*e.g.,* Adelman et al. (1983) *DNA* 2:183; Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) and can be achieved in a variety of ways generally known to those of skill in the art.

A.3. Other Structural Equivalents

The knowledge of the structure of the potassium-chloride cotransporter polypeptide of the present invention provides a means of investigating the mechanism of action of these proteins in a subject. For example, binding of these proteins to various substrate molecules can be predicted by various computer models. Upon discovering that such binding in fact takes place, knowledge of the protein structure then allows design and synthesis of small molecules, which mimic the functional binding of the potassium-chloride cotransporter polypeptide to the substrate. This is the method of "rational" drug design.

Use of the isolated and purified potassium-chloride cotransporter polypeptide of the present invention in rational drug design is thus contemplated in accordance with the present invention. Additional rational drug design techniques are described in U.S. Patent Nos. 5,834,228 and 5,872,011, herein incorporated in their entirety.

Thus, in addition to the peptidyl compounds described herein, the inventors also contemplate that other sterically similar compounds can be formulated to mimic the key portions of the peptide structure. Such compounds can be used in the same manner as the peptides of the invention and hence are also functional equivalents. The generation of a structural functional equivalent can be achieved by the techniques of modeling and chemical design known to those of skill in the art. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

B. Introduction of Gene Products

In accordance with the present invention, where a KCC gene itself is employed to introduce a KCC gene product, a convenient method of introduction will be through the use of a recombinant vector that incorporates the desired gene, together with its associated control sequences. The preparation of recombinant vectors is well known to those of skill in the art and described in many references, such as, for example, Sambrook et al., eds. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, incorporated herein in its entirety.

B.1. Vector Construction

It is understood that the DNA coding sequences to be expressed, in this case those encoding the potassium-chloride cotransporter gene products, are positioned in a vector adjacent to and under the control of a promoter. It is understood in the art that to bring a coding sequence under the control of such a promoter, one generally positions the 5′ end of the transcription initiation site of the transcriptional reading frame of the gene product to be expressed between about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3′ of) the chosen promoter.

One can also desire to incorporate into the transcriptional unit of the vector an appropriate polyadenylation site (*e.g.*, 5′-AATAAA-3′), if one was not contained within the original inserted DNA. Typically, these poly-A addition sites are placed about 30 to 2000 nucleotides "downstream" of the coding sequence at a position prior to transcription termination.

While use of the control sequences of the specific gene will be preferred, other control sequences can be employed, so long as they are compatible with the genotype of the cell being treated. Thus, one can mention other useful promoters by way of example, including, *e.g.*, an SV40 early promoter, a long terminal repeat promoter from retrovirus, an actin promoter, a heat shock promoter, a metallothionein promoter, and the like.

As is known in the art, a promoter is a region of a DNA molecule typically within about 100 nucleotide pairs upstream of (*i.e.*, 5' to) the point at which transcription begins (*i.e.*, a transcription start site). That region typically contains several types of DNA sequence elements that are located in similar relative positions in different genes.

Another type of discrete transcription regulatory sequence element is an enhancer. An enhancer imposes specificity of time, location and expression level on a particular coding region or gene. A major function of an enhancer is to increase the level of transcription of a coding sequence in a cell that contains one or more transcription factors that bind to that enhancer. An enhancer can function when located at variable distances from transcription start sites so long as a promoter is present.

As used herein, the phrase "enhancer-promoter" means a composite unit that contains both enhancer and promoter elements. An enhancer-promoter is operatively linked to a coding sequence that encodes at least one gene product. As used herein, the phrase "operatively linked" means that an enhancer-promoter is connected to a coding sequence in such a way that the transcription of that coding sequence is controlled and regulated by that enhancer-promoter. Means for operatively linking an enhancer-promoter to a coding sequence are well known in the art; the precise orientation and location

relative to a coding sequence of interest is dependent, inter alia, upon the specific nature of the enhancer-promoter.

An enhancer-promoter used in a vector construct of the present invention can be any enhancer-promoter that drives expression in a cell to be transfected. By employing an enhancer-promoter with well-known properties, the level and pattern of gene product expression can be optimized.

For introduction of, for example, a human potassium-chloride cotransporter gene, a vector construct that will deliver the gene to the affected cells is desired. Viral vectors can be used. These vectors will preferably be an adenoviral, a retroviral, a vaccinia viral vector, adeno-associated virus or Lentivirus; these vectors are preferred because they have been successfully used to deliver desired sequences to cells and tend to have a high infection efficiency. Suitable vector- potassium-chloride cotransporter gene constructs are adapted for administration as pharmaceutical compositions, as described herein below. Viral promoters can also be of use in vectors of the present invention, and are known in the art.

Commonly used viral promoters for expression vectors are derived from polyoma, cytomegalovirus, Adenovirus 2, and Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment that also contains the SV40 viral origin of replication. Smaller or larger SV40 fragments can also be used, provided there is included the approximately 250 base pair sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

The origin of replication can be provided either by construction of the vector to include an exogenous origin, such as can be derived from SV40 or other viral source, or can be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Where a potassium-chloride cotransporter gene itself is employed it will be most convenient to simply use a wild type potassium-chloride cotransporter gene directly. However, it is contemplated that certain regions of a potassium-chloride cotransporter gene can be employed exclusively without employing an entire wild type potassium-chloride cotransporter gene. It is proposed that it will ultimately be preferable to employ the smallest region needed to modulate biological activity so that one is not introducing unnecessary DNA into cells which receive a potassium-chloride cotransporter gene construct. The ability of these regions to modulate cell signaling can easily be determined by the assays reported in the Examples.

B.2. Transgenic Animals

It is also contemplated to be within the scope of the present invention to prepare a transgenic non-human animal that expresses a potassium-chloride cotransporter gene of the present invention. A preferred transgenic animal is a mouse.

Techniques for the preparation of transgenic animals are known in the art. Exemplary techniques are described in U.S. Patent No. 5,489,742 (transgenic rats); U.S. Patent Nos. 4,736,866, 5,550,316, 5,614,396, 5,625,125 and 5,648,061 (transgenic mice); U.S. Patent No. 5,573,933 (transgenic pigs); 5,162,215 (transgenic avian species) and U.S. Patent No. 5,741,957 (transgenic bovine species), the entire contents of each of which are herein incorporated by reference.

With respect to a representative method for the preparation of a transgenic mouse, cloned recombinant or synthetic DNA sequences or DNA segments encoding a potassium-chloride cotransporter gene product are injected into fertilized mouse eggs. The injected eggs are implanted in pseudo pregnant females and are grown to term to provide transgenic mice whose cells express a potassium-chloride cotransporter gene product.

For example, a transgenic animal of the present invention can comprises a mouse with targeted modification of the mouse KCC2, KCC3, and KCC4 genes. Mice strains with complete or partial functional inactivation of the KCC genes in all somatic cells are generated using standard techniques of site-

specific recombination in murine embryonic stem cells. See Capecchi (1989) *Science* 244(4910):1288-1292; Thomas & Capecchi (1990) *Nature* 346(6287):847-850; Delpire et al. (1999) *Nat Genet* 22(2):192-195. "knockout" murine KCC2 mice have been prepared, and the resultant homozygous $KCC2^{-/-}$ mice have a seizure disorder and increased perinatal mortality. The HCC2 knockout mice thus provide evidence that KCC2 is a drug target in epilepsy and further evidence that human KCC2 is a medically relevant gene.

Alternatives include the use of anti-sense or ribozyme KCC constructs, driven by a universal or tissue-specific promoter, to reduce levels of individual KCCs in somatic cells, thus achieving a "knock-down" of individual isoforms (Luyckx et al. (1999) *Proc Natl Acad Sci USA* 96(21):12174-12179). The invention also provides the generation of murine strains with conditional or inducible inactivation of individual or multiple KCC genes (Sauer (1998) *Methods* 14(4):381-392). For example, mice are created which lack expression of any KCCs in the renal proximal tubule, a known site of expression of KCC3 and KCC4, through the sequential mating of mice strains with lox-P-flanked KCC genes with a transgenic line expressing Cre-recombinase in the proximal tubule, using the promoter for the kidney androgen-regulated protein (Ding et al. (1997) *J Biol Chem* 272(44):28142-28148).

The present invention also provides mice strains with specific "knocked-in" modifications in the KCC2, KCC3, or KCC4 genes. This includes mice with genetically (Forlino et al. (1999) *J Biol Chem* 274(53):37923-37931) and functionally (Kissel et al. (2000) *EMBO J* 19(6):1312-1326) relevant point mutations in the KCC genes, in addition to manipulations such as the insertion of disease-specific repeat expansions (White et al. (1997) *Nat Genet* 17(4):404-410).

C. Generation of Antibodies

In still another embodiment, the present invention provides an antibody immunoreactive with a polypeptide of the present invention. Preferably, an antibody of the invention is a monoclonal antibody. Techniques for preparing and characterizing antibodies are well known in the art (See *e.g.,* Harlow &

Lane (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide or polynucleotide of the present invention, and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given polypeptide or polynucleotide can vary in its immunogenicity. It is often necessary therefore to couple the immunogen (*e.g.*, a polypeptide or polynucleotide) of the present invention) with a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers.

Means for conjugating a polypeptide or a polynucleotide to a carrier protein are well known in the art and include glutaraldehyde, NCmaleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, immunogenicity to a particular immunogen can be enhanced by the use of non-specific stimulators of the immune response known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen used of the production of polyclonal antibodies varies, inter alia, upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen, *e.g.,* subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal. The production of polyclonal antibodies is monitored by sampling blood of the immunized animal at various points following immunization. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored.

In another aspect, the present invention provides a process of producing an antibody immunoreactive with a potassium-chloride cotransporter polypeptide, the process comprising the steps of (a) transfecting recombinant host cells with a polynucleotide that encodes that polypeptide; (b) culturing the host cells under conditions sufficient for expression of the polypeptide; (c) recovering the polypeptide; and (d) preparing antibodies to the polypeptide. Preferably, the potassium-chloride cotransporter polypeptide is capable of modulating potassium and/or chloride levels within or outside of cells in accordance with the present invention.

A monoclonal antibody of the present invention can be readily prepared through use of well-known techniques such as the hybridoma techniques exemplified in U.S. Patent No 4,196,265 and the phage-displayed techniques disclosed in U.S. Patent No. 5,260,203, the contents of which are herein incorporated by reference.

A typical technique involves first immunizing a suitable animal with a selected antigen (*e.g.*, a polypeptide or polynucleotide of the present invention) in a manner sufficient to provide an immune response. Rodents such as mice and rats are preferred animals. Spleen cells from the immunized animal are then fused with cells of an immortal myeloma cell. Where the immunized animal is a mouse, a preferred myeloma cell is a murine NS-1 myeloma cell.

The fused spleen/myeloma cells are cultured in a selective medium to select fused spleen/myeloma cells from the parental cells. Fused cells are separated from the mixture of non-fused parental cells, for example, by the addition of agents that block the de novo synthesis of nucleotides in the tissue culture media. This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants for reactivity with an antigen-polypeptides. The selected clones can then be propagated indefinitely to provide the monoclonal antibody.

By way of specific example, to produce an antibody of the present invention, mice are injected intraperitoneally with between about 1-200 μg of an

antigen comprising a polypeptide of the present invention. B lymphocyte cells are stimulated to grow by injecting the antigen in association with an adjuvant such as complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*). At some time (*e.g.*, at least two weeks) after the first injection, mice are boosted by injection with a second dose of the antigen mixed with incomplete Freund's adjuvant.

A few weeks after the second injection, mice are tail bled and the sera titered by immunoprecipitation against radiolabeled antigen. Preferably, the process of boosting and titering is repeated until a suitable titer is achieved. The spleen of the mouse with the highest titer is removed and the spleen lymphocytes are obtained by homogenizing the spleen with a syringe.

Mutant lymphocyte cells known as myeloma cells are obtained from laboratory animals in which such cells have been induced to grow by a variety of well-known methods. Myeloma cells lack the salvage pathway of nucleotide biosynthesis. Because myeloma cells are tumor cells, they can be propagated indefinitely in tissue culture, and are thus "immortal". Numerous cultured cell lines of myeloma cells from mice and rats, such as murine NS-1 myeloma cells, have been established.

Myeloma cells are combined under conditions appropriate to foster fusion with the normal antibody-producing cells from the spleen of the mouse or rat injected with the antigen/polypeptide of the present invention. Fusion conditions include, for example, the presence of polyethylene glycol. The resulting fused cells are hybridoma cells. Like myeloma cells, hybridoma cells grow indefinitely in culture.

Hybridoma cells are separated from unfused myeloma cells by culturing in a selection medium such as HAT media (hypoxanthine, aminopterin, and thymidine). Unfused myeloma cells lack the enzymes necessary to synthesize nucleotides from the salvage pathway because they are killed in the presence of aminopterin, methotrexate, or azaserine. Unfused lymphocytes also do not continue to grow in tissue culture. Thus, only cells that have successfully fused (hybridoma cells) can grow in the selection media.

Each of the surviving hybridoma cells produces a single antibody. These cells are then screened for the production of the specific antibody immunoreactive with an antigen/polypeptide of the present invention. Single cell hybridomas are isolated by limiting dilutions of the hybridomas. The hybridomas are serially diluted many times and, after the dilutions are allowed to grow, the supernatant is tested for the presence of the monoclonal antibody. The clones producing that antibody are then cultured in large amounts to produce an antibody of the present invention in convenient quantity.

By use of a monoclonal antibody of the present invention, specific polypeptides and polynucleotide of the invention can be recognized as antigens, and thus identified. Once identified, those polypeptides and polynucleotide can be isolated and purified by techniques such as antibody-affinity chromatography. In antibody-affinity chromatography, a monoclonal antibody is bound to a solid substrate and exposed to a solution containing the desired antigen. The antigen is removed from the solution through an immunospecific reaction with the bound antibody. The polypeptide or polynucleotide is then easily removed from the substrate and purified.

D. <u>Detecting a Polynucleotide or a Polypeptide of the Present Invention</u>

Alternatively, the present invention provides a process of detecting a polypeptide of the present invention, wherein the process comprises immunoreacting the polypeptides with antibodies prepared according to the process described above to form antibody-polypeptide conjugates, and detecting the conjugates.

In yet another embodiment, the present invention provides a process of detecting messenger RNA transcripts that encode a polypeptide of the present invention, wherein the process comprises hybridizing the messenger RNA transcripts with polynucleotide sequences that encode the polypeptide to form duplexes; and detecting the duplex. Alternatively, the present invention provides a process of detecting DNA molecules that encode a polypeptide of the present invention, wherein the process comprises hybridizing DNA

molecules with a polynucleotide that encodes that polypeptide to form duplexes; and detecting the duplexes.

The detection and screening assays disclosed herein can optionally be used as a prognosis tool and/or diagnostic aid. KCC2-, KCC3-and/or KCC4-encoding polypeptides and nucleic acids can be readily used in clinical setting as a prognostic and/or diagnostic indicator for screening for levels of expression of potassium-chloride cotransporters, or alterations in native sequences. The nucleotide sequences of the subject invention can be used to detect differences in gene or gene product sequences between normal, carrier, or affected individuals. Such differences can consist of single-nucleotide changes or multiple changes, deletions, or additions in the native sequence which result in altered transcription, translation, or activity or biological activity or properties of the gene or gene product. These differences can be readily detected using the compositions of the present invention and techniques known in the art, including but not limited to SSCP analysis, RFLP analysis, or other PCR- or nucleotide-based analysis.

DNA segments of the invention or RNA having the sequence of, or a sequence complementary to, SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, or 112 can be used. Such polynucleic acids can comprise 10, 20, 40, 50, 70, 100, 250, 300, 400, 500, or 1,000 nucleotides or up to the full length of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, or 112. Such polynucleic acids can, but need not, encode polypeptides which retain some or all of the biological activity of the native gene or gene product.

D.1. Screening Assays for a Polypeptide of the Present Invention

The present invention provides a process of screening a biological sample for the presence of a potassium-chloride cotransporter polypeptide. A biological sample to be screened can be a biological fluid such as extracellular or intracellular fluid, or a cell or tissue extract or homogenate. A biological sample can also be an isolated cell (*e.g.*, in culture) or a collection of cells such as in a tissue sample or histology sample. A tissue sample can be suspended in a liquid medium or fixed onto a solid support such as a microscope slide. In accordance with a screening assay process, a biological sample is exposed to

an antibody immunoreactive with the polypeptide whose presence is being assayed. Typically, exposure is accomplished by forming an admixture in a liquid medium that contains both the antibody and the candidate polypeptide. Either the antibody or the sample with the polypeptide can be affixed to a solid support (*e.g.*, a column or a microtiter plate). Additional details of methods for such assays are known in the art. The presence of polypeptide in the sample is detected by evaluating the formation and presence of antibody-polypeptide conjugates. Techniques for detecting such antibody-antigen conjugates or complexes are well known in the art and include but are not limited to centrifugation, affinity chromatography and the like, and binding of a secondary antibody to the antibody-candidate receptor complex.

In one embodiment, detection is accomplished by detecting an indicator affixed to the antibody. Exemplary and well-known indicators include radioactive labels (*e.g.*, ^{32}P, ^{125}I, ^{14}C), a second antibody or an enzyme such as horseradish peroxidase. Techniques for affixing indicators to antibodies are known in the art.

In one embodiment, an antibody that recognizes a KCC4 polypeptide was used to detect KCC4 in mammalian tissues as described in Example 22. KCC4 is detected in a variety of tissues, including abundant expression in muscle, brain, lung, heart, and kidney. In another embodiment, an antibody that recognizes a KCC3 polypeptide was used to detect KCC3 in mammalian tissues as described in Examples 22-23. By performing this method, KCC3 isoforms KCC3a and KCC3b were distinguished by size. Further, the KCC3b isoform was shown to predominate in kidney, whereas KCC3a is predominate in the central nervous system. KCC3a is detected at the base of the choroid plexus epithelium, in large neurons, in the spinal dorsal and ventral columns, and in myelinated white matter tracts of the brain (Figures 29L-29M and 32). Differential expression of KCC isoforms KCC3a and KCC3b in brain and kidney, respectively, is also disclosed. KCC3a expression in brain supports a role of KCC3 in modulating neuronal communication and in controlling CNS excitability. Such a biological role is relevant to understanding, diagnosis, and treatment of epilepsy and other neural disorders, for example, Andermann's

syndrome. KCC3b expression in kidney points to a role in regulating hypertension, and other disorders of osmotic imbalance.

D.2. Screening Assay for Anti-Polypeptide Antibody

In another aspect, the present invention provides a process of screening a biological sample for the presence of antibodies immunoreactive with a potassium-chloride cotransporter polypeptide. Preferably the antibody so identified has activity in the modulation of potassium-chloride cotransporter biological activity in accordance with the present invention. In accordance with such a process, a biological sample is exposed to a KCC2, KCC3 and/or KCC4 polypeptide under biological conditions and for a period of time sufficient for antibody-polypeptide conjugate formation and the formed conjugates are detected.

D.3. Detection of a Polynucleotide That Encodes a KCC2, KCC3 and/or KCC4 Polypeptide of the Present Invention

A DNA or RNA molecule and particularly a DNA segment or polynucleotide can be used for hybridization to a DNA or RNA source or sample suspected of encoding a KCC polypeptide of the present invention; such molecules are referred to as "probes," and such hybridization is "probing". Such probes can be made synthetically. The probing is usually accomplished by hybridizing the oligonucleotide to a DNA source suspected of possessing a KCC (*e.g.*, KCC2, KCC3 and/or KCC4) gene. In some cases, the probes constitute only a single probe, and in others, the probes constitute a collection of probes based on a certain amino acid sequence or sequences of the polypeptide and account in their diversity for the redundancy inherent in the genetic code.

Other molecules which are neither DNA nor RNA but are capable of hybridizing in a similar manner and which are designed structurally to mimic the DNA or RNA sequence of a KCC (KCC2, KCC3 and/or KCC4) gene are also contemplated. Here, a suitable source to examine is capable of expressing a polypeptide of the present invention and can be a genomic library of a cell line of interest. Alternatively, a source of DNA or RNA can include total DNA or RNA from the cell line of interest. Once the hybridization process of the

invention has identified a candidate DNA segment, a positive clone can be confirmed by further hybridization, restriction enzyme mapping, sequencing and/or expression and testing.

Alternatively, such DNA molecules can be used in a number of techniques including their use as: (1) diagnostic tools to detect normal and abnormal DNA sequences in DNA derived from patient's cells; (2) reagents for detecting and isolating other members of the polypeptide family and related polypeptides from a DNA library potentially containing such sequences; (3) primers for hybridizing to related sequences for the purpose of amplifying those sequences; (4) primers for altering native potassium-chloride cotransporter DNA sequences; as well as (5) other techniques which rely on the similarity of the sequences of interest to those of the sequences herein disclosed.

As set forth above, in certain aspects, DNA sequence information provided by the invention allows for the preparation of probes that specifically hybridize to encoding sequences of a selected potassium-chloride cotransporter gene. In these aspects, probes of an appropriate length are prepared based on a consideration of the encoding sequence for a polypeptide of this invention. The ability of such probes to specifically hybridize to other encoding sequences lends them particular utility in a variety of embodiments. Most importantly, the probes can be used in a variety of assays for detecting the presence of complementary sequences in a given sample. However, other uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

To provide certain of the advantages in accordance with the invention, a preferred nucleic acid sequence employed for hybridization studies or assays includes probe sequences that are complementary to or mimic at least a 14 to 40 or so long nucleotide stretch of a nucleic acid sequence of the present invention, such as a sequence shown in any of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and 112. A size of at least 14 nucleotides in length helps to ensure that the fragment is of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches

greater than 14 bases in length are generally preferred, though, to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 14 to 20 nucleotides, or even longer where desired, such as 30, 40, 50, 60, 100, 200, 300, or 500 nucleotides or up to the full length of any of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and 112. Such fragments can be readily prepared by, for example, directly synthesizing the fragment by chemical synthesis, by application of nucleic acid amplification technology, such as the PCR technology of U.S. Patent No. 4,683,202, herein incorporated by reference, or by introducing selected sequences into recombinant vectors for recombinant production.

Accordingly, a nucleotide sequence of the present invention can be used for its ability to selectively form duplex molecules with complementary stretches of the gene. Depending on the application envisioned, one employs varying conditions of hybridization to achieve varying degrees of selectivity of the probe toward the target sequence. For applications requiring a high degree of selectivity, one typically employs relatively stringent conditions to form the hybrids. For example, one selects relatively low salt and/or high temperature conditions, such as provided by 0.02M-0.15M salt (*e.g.,* NaCl), including particularly 200mM salt, at temperatures of 50°C to 70°C, including particularly temperatures of about 55°C, about 60°C and about 65°C. Such conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

Of course, for some applications, less stringent hybridization conditions are typically needed to allow formation of the heteroduplex; one of skill in the art will know how to adjust the hybridization conditions for optimizing particular procedures. For example, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated by one

of skill in the art using known methods to carry out the desired function or experiment, without undue experimentation.

In one embodiment, the KCC sequences disclosed herein were used to detect a KCC RNA polynucleotide as described in Examples 19. By performing this method, KCC4 was determined to be expressed in a multitude of tissues, including robust expression in muscle, brain, lung, heart, and kidney. Expression of KCC4 in kidney supports a potential role in expression in kidney points to a role in regulating hypertension, and other disorders of osmotic imbalance.

In another embodiment, KCC3 was determined to be expressed in muscle, brain, lung, heart, and kidney. Differential expression of KCC isoforms KCC3a and KCC3b in brain and kidney, respectively, is also disclosed. KCC3a expression in brain supports a role of KCC3 in modulating neuronal communication and in controlling CNS excitability. Such a biological role is relevant to understanding, diagnosis, and treatment of epilepsy and other neural disorders, for example, Andermann's syndrome. KCC3b expression in kidney points to a role in regulating hypertension, and other disorders of osmotic imbalance.

D.4. Detection Assay Kits

In another aspect, the present invention provides assay kits for detecting the presence of a polypeptide of the present invention in biological samples, where the kits comprise a first antibody capable of immunoreacting with the polypeptide. Preferably, the assay kits of the invention further comprise a second container containing a second antibody that immunoreacts with the first antibody. More preferably, the antibodies used in the assay kits of the present invention are monoclonal antibodies. Even more preferably, the first antibody is affixed to a solid support. More preferably still, the first and second antibodies comprise an indicator, and, preferably, the indicator is a radioactive label or an enzyme.

The present invention also provides an assay kit for screening agents. Such a kit can contain a polypeptide of the present invention. The kit can

additionally contain reagents for detecting an interaction between an agent and a polypeptide of the present invention.

In an alternative aspect, the present invention provides assay kits for detecting the presence, in biological samples, of a polynucleotide that encodes a polypeptide of the present invention, the kits comprising a first container that contains a second polynucleotide identical or complementary to a segment of at least 10 contiguous nucleotide bases of, as a preferred example, any of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and 112. In another embodiment, the present invention provides assay kits for detecting the presence, in a biological sample, of antibodies immunoreactive with a polypeptide of the present invention, the kits comprising a KCC (*e.g.,* KCC2, KCC3 and/or KCC4) polypeptide that immunoreacts with the antibodies.

E. Mapping and Polynucleotide Screening

In another embodiment of the invention, the nucleic acid sequences that encode KCC2, KCC3 and/or KCC4 can also be used to generate hybridization probes which are useful for mapping naturally occurring genomic sequences and/or disease loci. The sequences can be mapped to a particular chromosome or to a specific region of the chromosome using well-known techniques. Such techniques include FISH, FACS, or artificial chromosome constructions, such as yeast artificial chromosomes, bacterial artificial chromosomes, bacterial P1 constructions or single chromosome cDNA libraries as reviewed in Price (1993) *Blood Rev* 7:127-134, and Trask (1991) *Trends Genet* 7:149-154.

FISH (as described in Verma et al. (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York, New York) can be correlated with other physical chromosome mapping techniques and genetic map data. Examples of genetic map data can be found in the 1994 Genome Issue of *Science* (265:1981f). Correlation between the location of the gene encoding KCC2, KCC3 and/or KCC4 on a physical chromosomal map and a specific disease, or predisposition to a specific disease, can help delimit the region of DNA associated with that genetic disease. The nucleotide sequences

of the subject invention can be used to detect differences in gene sequences between normal, carrier, or affected individuals.

In situ hybridization of chromosomal preparations and physical mapping techniques such as linkage analysis using established chromosomal markers can be used for extending genetic maps. Often the placement of a gene on the chromosome of another mammalian species, such as mouse, reveals associated markers also found in other mammals such as humans even if the number or arm of a particular human chromosome is not known. New sequences can be assigned to chromosomal arms, or parts thereof, by physical mapping. This provides valuable information to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once the disease or syndrome has been crudely localized by genetic linkage to a particular genomic region, for example, ataxia-telangiectasia (A-T) to 11q22-23 (Gatti et al. (1988) *Nature* 336:577-580), any sequences mapping to that area can represent associated or regulatory genes for further investigation. The nucleotide sequences of the present invention can thus also be used to detect differences in the chromosomal location due to translocation, inversion, *etc.* among normal, carrier, or affected individuals.

The mapping methods of the present invention also employ genomic clones of the exons of KCC2, KCC3 and KCC4. Coding and genomic sequences for human KCC2 are set forth in SEQ ID NOs: 20-63. Coding sequences from human KCC2 exons 1-24 (SEQ ID NOs:20-43) are set forth herein. Genomic sequences for human KCC2 exons 2-7 (SEQ ID NOs:44-49), exons 9-14 (SEQ ID NOs:50-55), and exons 17-24 (SEQ ID NOs:56-63) are set forth herein.

Genomic sequences for human KCC4 are set forth in SEQ ID NOs: 64-83. Genomic sequences for human KCC4 exon 2 (SEQ ID NO:64), exon 3 (SEQ ID NO:65), exon 5 (SEQ ID NO:66), exons 6-10 (SEQ ID NOs:67-71), exons 12-19 (SEQ ID NOs:72-79), and exons 21-24 (SEQ ID NOs:80-83) are set forth herein.

Genomic sequences for human KCC3a and human KCC3b are set forth in SEQ ID NOs:84-110. Genomic sequences for human KCC3 exon 1 -

hKCC3a (SEQ ID NO:84), exon 1 - hKCC3b (SEQ ID NO:85), exon 2 (SEQ ID NO:86), exon 3 (SEQ ID NO:87), exon 4 - HKCC3b (SEQ ID NO:88), exon 4 - HKCC3a (SEQ ID NO:89), exons 6-11 (SEQ ID NOs:90-95), exon 12 - hKCC3b (SEQ ID NO:96), exon 12 - hKCC3a (SEQ ID NO:97) and exons 13-25 (SEQ ID NOs:98-110) are set forth herein.

In another embodiment, the present invention provides genetic assays based on the genomic sequence of the human KCC2, KCC3, and KCC4 genes. The intronic sequence flanking the individual exons encoding the three genes, described as SEQ ID NOs:44-110, is employed in the design of oligonucleotide primers suitable for the mutation analysis of human genomic DNA. Thus, intronic primers can be used to screen for genetic variants by a number of PCR-based techniques, including single-strand conformation polymorphism (SSCP) analysis (Orita et al. (1989) *Proc Natl Acad Sci USA* 86(8):2766-2770), SSCP/heteroduplex analysis, enzyme mismatch cleavage, and direct sequence analysis of amplified exons (Kestila et al. (1998) *Mol Cell* 1(4), 575-582; Yuan et al. (1999) *Hum Mutat* 14(5):440-446).

Similar techniques can be applied to putative 5'-regulatory regions, *e.g.,* the putative promoters 5' of exons 1a and 1b of human or mouse KCC3 (*e.g.,* SEQ ID NOs:17-19). Automated methods can also be applied the large-scale characterization of single nucleotide polymorphisms (Brookes (1999) *Gene* 234(2):177-186; Wang et al. (1998) *Science* 280(5366):1077-1082) within and near the human KCC genes. Once genetic variants have been detected in specific patient populations, *e.g.,* KCC3 mutations in patients with Andermann's syndrome, the present invention provides assays to detect the mutation by methods such as allele-specific hybridization (Stoneking et al. (1991) *Am J Hum Genet* 48(2):370-382), or restriction analysis of amplified genomic DNA containing the specific mutation. Again, these detection methods can be automated using existing technology (Wang et al. (1998) *Science* 280(5366):1077-1082). In the case of genetic disease or human phenotypes caused by repeat expansion (Lafreniere et al. (1997) *Nat Genet* 15(3):298-302; Timchenko & Caskey (1996) *FASEB J* 10(14):1589-1597), the invention

provides an assay based on PCR of genomic DNA with oligonucleotide primers flanking the involved repeat.

As used herein and in the claims, the term "polymorphism" refers to the occurrence of two or more genetically determined alternative sequences or alleles in a population. A polymorphic marker is the locus at which divergence occurs. Preferred markers have at least two alleles, each occurring at frequency of greater than 1%. A polymorphic locus can be as small as one base pair.

As used herein and in the claims, the term "gene" is used for simplicity to refer to a functional protein, polypeptide or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences and cDNA sequences. "Isolated substantially away from other coding sequences" means that the gene of interest, in this case, a KCC gene, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

The provided nucleic acid molecules can be labeled according to any technique known in the art, such as with radiolabels, fluorescent labels, enzymatic labels, sequence tags, *etc.* Such molecules can be used as allele-specific oligonucleotide probes. Body samples can be tested to determine whether a KCC gene contains a polymorphism. Suitable body samples for testing include those comprising DNA, RNA or protein obtained from biopsies, including liver and intestinal tissue biopsies; or from blood, prenatal; or embryonic tissues, for example.

In one embodiment of the invention two pairs of isolated oligonucleotide primers are provided. These sets of primers are optionally derived from a KCC exon. The oligonucleotide primers are useful, for example, in detecting a polymorphism of a KCC gene. The primers direct amplification of a target polynucleotide prior to sequencing. In another embodiment of the invention

isolated allele specific oligonucleotides (ASO) are provided. The allele specific oligonucleotides are also useful in detecting a polymorphism of a KCC gene.

The terms "substantially complementary to" or "substantially the sequence of" refer to sequences which hybridize to the sequences provided (*e.g.,* SEQ ID NO:44-110) under stringent conditions as disclosed herein and/or sequences having sufficient homology with any of SEQ ID NOs: 44-110, such that the allele specific oligonucleotides of the invention hybridize to the sequence. The term "isolated" as used herein includes oligonucleotides substantially free of other nucleic acids, proteins, lipids, carbohydrates or other materials with which they can be associated, such association being either in cellular material or in a synthesis medium. A "target polynucleotide" or "target nucleic acid" refers to the nucleic acid sequence of interest *e.g.*, a KCC-encoding polynucleotide. Other primers which can be used for primer hybridization are readily ascertainable to those of skill in the art based upon the disclosure herein.

The primers of the invention embrace oligonucleotides of sufficient length and appropriate sequence so as to provide initiation of polymerization on a significant number of nucleic acids in the polymorphic locus. Specifically, the term "primer" as used herein refers to a sequence comprising two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and more preferably more than eight and most preferably at least about 20 nucleotides of a KCC exonic or intronic region as are disclosed herein. Such oligonucleotides are preferably between ten and thirty bases in length. Such oligonucleotides can optionally further comprise a detectable label.

Environmental conditions conducive to synthesis include the presence of nucleoside triphosphates and an agent for polymerization, such as DNA polymerase, and a suitable temperature and pH. The primer is preferably single stranded for maximum efficiency in amplification, but can be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent for polymerization. The exact length of primer will depend

on many factors, including temperature, buffer, and nucleotide composition. The oligonucleotide primer typically contains 12-20 or more nucleotides, although it can contain fewer nucleotides.

Primers of the invention are designed to be "substantially" complementary to each strand of the genomic locus to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands under conditions that allow the agent for polymerization to perform. In other words, the primers should have sufficient complementarity with the 5' and 3' sequences flanking the transition to hybridize therewith and permit amplification of the genomic locus.

Oligonucleotide primers of the invention are employed in the amplification method that is an enzymatic chain reaction that produces exponential quantities of polymorphic locus relative to the number of reaction steps involved. Typically, one primer is complementary to the negative (-) strand of the polymorphic locus and the other is complementary to the positive (+) strand. Annealing the primers to denatured nucleic acid followed by extension with an enzyme, such as the large fragment of DNA polymerase I (Klenow) and nucleotides, results in newly synthesized + and - strands containing the target polymorphic locus sequence. Because these newly synthesized sequences are also templates, repeated cycles of denaturing, primer annealing, and extension results in exponential production of the region (*i.e.*, the target polymorphic locus sequence) defined by the primers. The product of the chain reaction is a discreet nucleic acid duplex with termini corresponding to the ends of the specific primers employed.

The oligonucleotide primers of the invention can be prepared using any suitable method, such as conventional phosphotriester and phosphodiester methods or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and can be synthesized as described by Beaucage et al. (1981) *Tetrahedron Letters* 22:1859-1862. One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Patent No. 4,458,066.

Any nucleic acid specimen, in purified or non-purified form, can be utilized as the starting nucleic acid or acids, providing it contains, or is suspected of containing, a nucleic acid sequence containing the polymorphic locus. Thus, the method can amplify, for example, DNA or RNA, including messenger RNA, wherein DNA or RNA can be single stranded or double stranded. In the event that RNA is to be used as a template, enzymes, and/or conditions optimal for reverse transcribing the template to DNA would be utilized. In addition, a DNA-RNA hybrid that contains one strand of each can be utilized. A mixture of nucleic acids can also be employed, or the nucleic acids produced in a previous amplification reaction herein, using the same or different primers can be so utilized. The specific nucleic acid sequence to be amplified, *i.e.*, the polymorphic locus, can be a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified is present initially in a pure form; it can be a minor fraction of a complex mixture, such as contained in whole human DNA.

DNA utilized herein can be extracted from a body sample, such as blood, tissue material (*e.g.,* brain or kidney tissue), and the like by a variety of techniques such as that described by Maniatis et. al. (1982) in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, New York. If the extracted sample is impure, it can be treated before amplification with an amount of a reagent effective to open the cells, or animal cell membranes of the sample, and to expose and/or separate the strand(s) of the nucleic acid(s). This lysing and nucleic acid denaturing step to expose and separate the strands will allow amplification to occur much more readily.

The deoxyribonucleotide triphosphates dATP, dCTP, dGTP, and dTTP are added to the synthesis mixture, either separately or together with the primers, in adequate amounts and the resulting solution is heated to about 90-100°C from about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period, the solution is allowed to cool, which is preferable for the primer hybridization. To the cooled mixture is added an appropriate agent for effecting the primer extension reaction (called herein "agent for polymerization"), and the

reaction is allowed to occur under conditions known in the art. The agent for polymerization can also be added together with the other reagents if it is heat stable. This synthesis (or amplification) reaction can occur at room temperature up to a temperature above which the agent for polymerization no longer functions. Thus, for example, if DNA polymerase is used as the agent, the temperature is generally no greater than about 40°C. Most conveniently the reaction occurs at room temperature.

The agent for polymerization can be any compound or system that will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase, polymerase muteins, reverse transcriptase, other enzymes, including heat-stable enzymes (*i.e.*, those enzymes which perform primer extension after being subjected to temperatures sufficiently elevated to cause denaturation), such as Taq polymerase. Suitable enzyme will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each polymorphic locus nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths.

The newly synthesized strand and its complementary nucleic acid strand will form a double-stranded molecule under hybridizing conditions described herein and this hybrid is used in subsequent steps of the method. In the next step, the newly synthesized double-stranded molecule is subjected to denaturing conditions using any of the procedures described above to provide single-stranded molecules.

The steps of denaturing, annealing, and extension product synthesis can be repeated as often as needed to amplify the target polymorphic locus nucleic acid sequence to the extent necessary for detection. The amount of the specific nucleic acid sequence produced will accumulate in an exponential fashion. See McPherson et al., eds. (1991) PCR. A Practical Approach, IRL Press, Oxford University Press, New York, New York.

The amplification products can be detected by Southern blot analysis with or without using radioactive probes. In one such method, for example, a small sample of DNA containing a very low level of the nucleic acid sequence of the polymorphic locus is amplified, and analyzed via a Southern blotting technique or similarly, using dot blot analysis. The use of non-radioactive probes or labels is facilitated by the high level of the amplified signal. Alternatively, probes used to detect the amplified products can be directly or indirectly detectably labeled, for example, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Those of ordinary skill in the art will know of other suitable labels for binding to the probe, or will be able to ascertain such, using routine experimentation.

Sequences amplified by the methods of the invention can be further evaluated, detected, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence such as dideoxy sequencing, PCR, oligomer restriction (Saiki et al. (1985) *Bio/Technology* 3:1008-1012), allele-specific oligonucleotide (ASO) probe analysis (Conner et al. (1983) *Proc Natl Acad Sci USA* 80:278), oligonucleotide ligation assays (OLAs) (Landgren et al. (1988) *Science* 241:1007), and the like. Molecular techniques for DNA analysis have been reviewed (Landgren et al. (1988) *Science* 242:229-237).

Preferably, the method of amplifying is by PCR, as described herein and in U.S. Patent Nos. 4,683,195; 4,683,202; and 4,965,188 each of which is hereby incorporated by reference; and as is commonly used by those of ordinary skill in the art. Alternative methods of amplification have been described and can also be employed as long as a KCC locus amplified by PCR using primers of the invention is similarly amplified by the alternative means. Such alternative amplification systems include but are not limited to self-sustained sequence replication, which begins with a short sequence of RNA of interest and a T7 promoter. Reverse transcriptase transcribes the RNA into cDNA and degrades the RNA, followed by reverse transcriptase polymerizing a second strand of DNA.

Another nucleic acid amplification technique is nucleic acid sequence-based amplification (NASBA™) which uses reverse transcription and T7 RNA polymerase and incorporates two primers to target its cycling scheme. NASBA™ amplification can begin with either DNA or RNA and finish with either, and amplifies to about 108 copies within 60 to 90 minutes.

Alternatively, nucleic acid can be amplified by ligation-activated transcription (LAT). LAT works from a single-stranded template with a single primer that is partially single-stranded and partially double-stranded. Amplification is initiated by ligating a cDNA to the promoter oligonucleotide and within a few hours, amplification is about 108 to about 109 fold. The QB replicase system can be utilized by attaching an RNA sequence called MDV-1 to RNA complementary to a DNA sequence of interest. Upon mixing with a sample, the hybrid RNA finds its complement among the specimen's mRNAs and binds, activating the replicase to copy the tag-along sequence of interest.

Another nucleic acid amplification technique, ligase chain reaction (LCR), works by using two differently labeled halves of a sequence of interest that are covalently bonded by ligase in the presence of the contiguous sequence in a sample, forming a new target. The repair chain reaction (RCR) nucleic acid amplification technique uses two complementary and target-specific oligonucleotide probe pairs, thermostable polymerase and ligase, and DNA nucleotides to geometrically amplify targeted sequences. A 2-base gap separates the oligo probe pairs, and the RCR fills and joins the gap, mimicking normal DNA repair.

Nucleic acid amplification by strand displacement activation (SDA) utilizes a short primer containing a recognition site for Hinc II with short overhang on the 5' end which binds to target DNA. A DNA polymerase fills in the part of the primer opposite the overhang with sulfur-containing adenine analogs. Hinc II is added but only cuts the unmodified DNA strand. A DNA polymerase that lacks 5' exonuclease activity enters at the site of the nick and begins to polymerize, displacing the initial primer strand downstream and building a new one which serves as more primer.

SDA produces greater than about a 10^7-fold amplification in 2 hours at 37°C. Unlike PCR and LCR, SDA does not require instrumented temperature cycling. Another amplification system useful in the method of the invention is the QB Replicase System. Although PCR is the preferred method of amplification if the invention, these other methods can also be used to amplify the KCC locus as described in the method of the invention. Thus, the term "amplification technique" as used herein and in the claims is meant to encompass all the foregoing methods.

In another embodiment of the invention a method is provided for identifying a subject having a polymorphism of a KCC gene, comprising sequencing a target nucleic acid of a sample from a subject by dideoxy sequencing, preferably following amplification of the target nucleic acid.

In another embodiment of the invention a method is provided for identifying a subject having a polymorphism of a KCC gene, comprising contacting a target nucleic acid of a sample from a subject with a reagent that detects the presence of a KCC polymorphism and detecting the reagent. A number of hybridization methods are well known to those skilled in the art. Many of them are useful in carrying out the invention.

Nucleic acid hybridization will be affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of nucleotide base mismatches between the hybridizing nucleic acids, as will be readily appreciated by those of ordinary skill in the art. Stringent temperature conditions will generally include temperatures in excess of 30°C, typically in excess of 37°C, and preferably in excess of 45°C. Stringent salt conditions will ordinarily be less than 1,000mM, typically less than 500mM, and preferably less than 200mM. However, the combination of parameters is much more important than the measure of any single parameter. See, *e.g.,* Wethmur & Davidson (1986) *J Mol Biol* 31:349-370.

Accordingly, a nucleotide sequence of the present invention can be used for its ability to selectively form duplex molecules with complementary stretches of a KCC gene. Depending on the application envisioned, one employs varying

conditions of hybridization to achieve varying degrees of selectivity of the probe toward the target sequence. For applications requiring a high degree of selectivity, one typically employs relatively stringent conditions to form the hybrids. For example, one selects relatively low salt and/or high temperature conditions, such as provided by 0.02M-0.15M salt at temperatures of about 50°C to about 70°C including particularly temperatures of about 55°C, about 60°C and about 65°C. Such conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate polypeptide coding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions are typically needed to allow formation of the heteroduplex. Under such circumstances, one employs conditions such as 0.15M-0.9M salt, at temperatures ranging from about 20°C to about 55°C, including particularly temperatures of about 25°C, about 37°C, about 45°C, and about 50°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it is advantageous to employ a nucleic acid sequence of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator reagents are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one likely employs an enzyme tag such a urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, calorimetric indicator substrates are known which can be employed to provide a reagent

visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein are useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the sample containing test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions depend inter alia on the particular circumstances based on the particular criteria required (depending, for example, on the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label.

The materials for use in the method of the invention are ideally suited for the preparation of a screening kit. Such a kit can comprise a carrier having compartments to receive in close confinement one or more containers such as vials, tubes, and the like, each of the containers comprising one of the separate elements to be used in the method. For example, one of the containers can comprise an amplifying reagent for amplifying a KCC DNA, such as the necessary enzyme(s) and oligonucleotide primers for amplifying target DNA from the subject.

The oligonucleotide primers include primers having a sequence derived from the group including, but not limited to: SEQ ID NOs:44-110, or primer sequences substantially complementary or substantially homologous thereto. Oligonucleotide primers comprising target flanking 5' and 3' polynucleotide sequence have substantially the sequence set forth in the flanking 5' and 3' portions of any of SEQ ID NOs: 1-16, 44-110, 112-113, and sequences substantially complementary or homologous thereto. Other oligonucleotide primers for amplifying a KCC will be known or readily ascertainable to those of skill in the art given the disclosure of the present invention presented herein.

A kit in accordance with the present invention can further comprise solutions, buffers or other reagents for extracting a nucleic acid sample from a biological sample obtained from a subject. Any such reagents as would be readily apparent to one of ordinary skill in the art is contemplated to fall within the scope of the present invention. By way of particular example, a suitable lysis buffer for the tissue or cells along with a suspension of glass beads for capturing the nucleic acid sample and an elution buffer for eluting the nucleic acid sample off of the glass beads comprise a reagent for extracting a nucleic acid sample from a biological sample obtained from a subject.

Other examples include commercially available extraction kits, such as the GENOMIC ISOLATION KIT A.S.A.P.™ (Boehringer Mannheim of Indianapolis, Indiana), Genomic DNA Isolation System (GIBCO BRL of Gaithersburg, Maryland), ELU-QUIK™ DNA Purification Kit (Schleicher & Schuell of Keene, New Hampshire), DNA Extraction Kit (Stratagene of La Jolla, California), TURBOGEN™ Isolation Kit (Invitrogen of San Diego, California), and the like. Use of these kits according to the manufacturer's instructions is generally acceptable for purification of DNA prior to practicing the methods of the present invention.

F. Screening for Modulators of KCC Biological Activity

In yet another aspect, the present invention provides a process of screening substances for their ability to affect or modulate the biological activity of potassium-chloride cotransporter gene products, and for their ability to affect or to modulate *in vivo* potassium-chloride cotransporter levels. The present invention also provides a process of screening substances for their ability to affect or modulate the biological activity of KCC2, KCC3 and/or KCC4 gene products, and for their ability to affect or modulate *in vivo* KCC2, KCC3 and/or KCC4 levels. This modulation can affect cell growth and differentiation.

Utilizing the methods and compositions of the present invention, screening assays for the testing of candidate substances are performed. A candidate substance is a substance which potentially can promote or inhibit the

biological activity of gene product by binding or other intermolecular interaction with the KCC2, KCC3 and/or KCC4 gene or gene product or control sequence.

F.1. Method of Screening for Modulators of KCC2, KCC3 and/or KCC4 Biological Activity

A representative method of screening candidate substances for their ability to modulate KCC biological activity comprises: (a) establishing replicate test and control samples that comprise a biologically active KCC polypeptide; (b) administering a candidate substance to test samples; (c) measuring the biological activity of the KCC polypeptide in the test and the control samples; and (d) determining whether the candidate substance modulates KCC biological activity relative to an appropriate control. By "modulate" is intended an increase, decrease, or other alteration of any or all biological activities or properties of KCC. A candidate substance identified according to the screening assay described herein has an ability to modulate KCC biological activity. Such a candidate compound has utility in the treatment of disorders and conditions associated with the biological activity of a KCC, such as KCC2, KCC3 and/or KCC4. Candidate compounds are typically about 500-1000 daltons, and can be hydrophobic, polycyclic, or both, molecules.

In a cell-free system, the method comprises the steps of establishing a control system comprising a KCC polypeptide and a ligand to which the KCC polypeptide is capable of binding, establishing a test system comprising the KCC polypeptide, the ligand, and a candidate compound, and determining whether the candidate compound modulates KCC activity in a cell-free system. A representative ligand comprises a monoclonal antibody, and in this embodiment, the biological activity or property screened includes binding affinity.

In another embodiment of the invention, a KCC polypeptide (*e.g.*, KCC2, KCC3 and/or KCC4) or catalytic or immunogenic fragment or oligopeptide thereof, can be used for screening libraries of compounds in any of a variety of drug screening techniques. The fragment employed in such screening can be free in solution, affixed to a solid support, borne on a cell surface, or located

intracellularly. The formation of binding complexes, between the KCC polypeptide and the agent being tested, can be measured.

Another technique for drug screening that can be used provides for high throughput screening of compounds having suitable binding affinity to the protein of interest as described in published International Publication Number WO 84/03564, herein incorporated by reference. In this method, as applied to the KCC polypeptide, large numbers of different small test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The test compounds are reacted with the KCC polypeptide, or fragments thereof, and washed. Bound KCC polypeptide is then detected by methods well known in the art. Purified KCC polypeptide can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

A screening assay of the present invention can also involve determining the ability of a candidate substance to modulate, *i.e.* inhibit or promote KCC biological activity and preferably, to thereby modulate the biological activity of potassium-chloride cotransporters in target cells. Target cells can be either naturally occurring cells known to contain a polypeptide of the present invention or transformed cells produced in accordance with a process of transformation set forth herein above. The test samples can further comprise a cell or cell line that expresses a KCC polypeptide; the present invention also provides a recombinant cell line suitable for use in the exemplary method. Such cell lines can be mammalian, or human, or they can from another organism, including but not limited to yeast. Exemplary assays including genetic screening assays and molecular biology screens such as a yeast two-hybrid screen that will effectively identify KCC-interacting genes important for potassium-chloride cotransport or other KCC-mediated cellular process. One version of the yeast two-hybrid system has been described (Chien et al. (1991) *Proc Natl Acad Sci USA*, 88:9578-9582) and is commercially available from Clontech (Palo Alto, California).

A preferred screening system for the measurement of K^+- Cl^- cotransport activity is the injection of cRNA (copy RNA) encoding individual KCC isoforms into *Xenopus* laevis oocytes, as described in Example 11. Indeed, the present invention provides the first disclosure of the use of oocytes for K^+- Cl^- cotransporters. A significant advantage of *Xenopus* oocytes is the ability to express multiple different constructs in a minimum of time. The endogenous K^+- Cl^- cotransport activity is also negligible, which facilitates the analysis of KCC constructs. A drawback is the oocyte-to-oocyte, experiment-to-experiment, and frog-to-frog variability. However, comparing multiple constructs and conditions in the same experiments can compensate for this variability. Moreover, *Xenopus* oocytes have been used successfully for the kinetic analysis of cation-chloride cotransporters (Gamba et al. (1993) *Proc Natl Acad Sci USA* 90(7):2749-2753; Giménez et al. (1999) *FASEB J* 13:A64). A number of other volume-sensitive transporters, ion channels and related proteins have been expressed in *Xenopus* oocytes, with the appropriate physiological response (Ji et al. (1998) *Am J Physiol* 275(5 Pt 1):C1182-1190; Krapivinsky et al. (1994) *Cell* 76(3):439-448; Vandorpe et al. (1998) *J Biol Chem* 273(34):21542-21553; and Grunder et al. (1992) *Nature* 360(6406):759-762). Thus the Na^+-K^+-2 Cl^- cotransporter BSC2/NKCC1 is activated by hypertonicity when expressed in oocytes, as they are in mammalian cells. Cell swelling also activates the major red cell K^+- Cl^- cotransporter KCC1 (Su et al. (1999) *Am J Physiol* 277(5 Pt 1):C899-C912) and the $Ca2^+$-activated K^+ channel ISK1 (Vandorpe et al. (1998) *J Biol Chem* 273(34):21542-53) expressed in oocytes, as predicted by their behavior in red cells.

As is well known in the art, a screening assay can provide a cell under conditions suitable for testing the modulation of KCC biological activity and/or levels of potassium-chloride cotransporters. These conditions include but are not limited to pH, temperature, tonicity, the presence of relevant metabolic factors (*e.g.*, metal ions such as for example Ca^{++}, growth factor, interleukins, or colony stimulating factors), and relevant modifications to the polypeptide such as glycosylation or prenylation. It is contemplated that a polypeptide of the present invention can be expressed and utilized in a prokaryotic or eukaryotic

cell. The host cell can also be fractionated into sub-cellular fractions where the receptor can be found. For example, cells expressing the polypeptide can be fractionated into the nuclei, the endoplasmic reticulum, vesicles, or the membrane surfaces of the cell. U.S. Patent Nos. 5,837,479; 5,645,999; 5,786,152; 5,739,278; and 5,352,660 also describe exemplary screening assays, and the entire contents of each are herein incorporated by reference.

In one embodiment, a screening assay is designed to be capable of discriminating candidate substances having selective ability to interact with or modulate one or more of the genes or gene products of the present invention but which substances are without a substantially overlapping activity with another gene or gene product. For example, a substance can modulate the biological activity of KCC3 but have no effect, or a diminished effect, on KCC4. Such selective effect can consist of a 30% greater effect on one test sample versus another, or more preferably 100% or greater effect.

A method of identifying modulators of potassium-chloride cotransporters by rational drug design is contemplated in accordance with the present invention. The method comprises the steps of designing a potential modulator for a potassium-chloride cotransporter that will form non-covalent bonds with amino acids in the substrate binding site based upon the structure of a KCC (*e.g.*, KCC2, KCC3 and/or KCC4) polypeptide; synthesizing the modulator; and determining whether the potential modulator modulates the activity of a potassium-chloride cotransporter. Modulators can be synthesized using techniques known in the art. The determination of whether the modulator modulates the biological activity of a potassium-chloride cotransporter is made in accordance with the screening methods disclosed herein, or by other screening methods known in the art.

F.2 Method of Screening for Modulators of *In vivo* Potassium-Chloride Cotransporter Levels

In accordance with the present invention there are also provided methods for screening candidate compounds for the ability to modulate *in vivo* potassium-chloride cotransporter levels and/or activity. Representative modulators of KCC (*e.g.*, KCC2, KCC3 and/or KCC4) levels can comprise

modulators of potassium-chloride cotransporter transcription or expression. Pharmaceuticals that increase or decrease the transcription or expression of potassium-chloride cotransporter encoding genes have important clinical application for the modulation of the biological activity of potassium-chloride cotransporters. This modulation can affect potassium-chloride homeostasis.

This invention thus includes a method for discovery of compounds that modulate the expression levels of potassium-chloride cotransporter encoding genes, including not only the KCC2, KCC3 and/or KCC4 of the present invention but also other potassium-chloride cotransporter-encoding genes, and describes the use of such compounds. The general approach is to screen compound libraries for substances which increase or decrease expression of KCC2-, KCC3- and/or KCC4-encoding genes. Exemplary techniques are described in U.S. Patent Nos. 5,846,720 and 5,580,722, the entire contents of each of which are herein incorporated by reference.

While the following terms are believed to be well understood by one of skill in the art, the following definitions are set forth to facilitate explanation of the invention.

"Transcription" means a cellular process involving the interaction of an RNA polymerase with a gene that directs the expression as RNA of the structural information present in the coding sequences of the gene. The process includes, but is not limited to the following steps: (a) the transcription initiation, (b) transcript elongation, (c) transcript splicing, (d) transcript capping, (e) transcript termination, (f) transcript polyadenylation, (g) nuclear export of the transcript, (h) transcript editing, and (i) stabilizing the transcript. "Expression" generally refers to the cellular processes by which a biologically active polypeptide is produced from RNA.

"Transcription factor" means a cytoplasmic or nuclear protein which binds to such gene, or binds to an RNA transcript of such gene, or binds to another protein which binds to such gene or such RNA transcript or another protein which in turn binds to such gene or such RNA transcript, so as to thereby modulate expression of the gene. Such modulation can additionally be

achieved by other mechanisms; the essence of "transcription factor for a gene" is that the level of transcription of the gene is altered in some way.

In accordance with the present invention there is provided a method of identifying a candidate compound or molecule that is capable of modulating the transcription level of a gene encoding a potassium-chloride cotransporter and thus is capable of acting as a therapeutic agent in the modulation of potassium-chloride cotransporter effects. This modulation can affect cell growth and differentiation. Such modulation can be direct, *i.e.*, through binding of a candidate molecule directly to the nucleotide sequence, whether DNA or RNA transcript, or such modulation can be achieved via one or more intermediaries, such as proteins other than KCC2, KCC3 and/or KCC4 which are affected by the candidate compound and ultimately modulate potassium-chloride cotransporter transcription by any mechanism, including direct binding, phosphorylation or dephosphorylation, *etc.*

This method comprises contacting a cell or nucleic acid sample with a candidate compound or molecule to be tested. These samples contain nucleic acids which can contain elements that modulate transcription and/or translation of the KCC2, KCC3 and/or KCC4 gene, such as a KCC2, KCC3 or KCC4 promoter or putative upstream regulatory region (representative promoters disclosed herein as SEQ ID NOs:17-19 and 131), and a DNA sequence encoding a polypeptide which can be detected in some way. Thus, the polypeptide can be described as a "reporter" or "marker." Preferably, the candidate compound directly and specifically transcriptionally modulates expression of the potassium-chloride-cotransporter-encoding gene. Such compounds are anticipated to have therapeutic or pharmaceutical uses in treating potassium-chloride-cotransporter-related diseases and/or disorders.

The DNA sequence is coupled to and under the control of the promoter, under conditions such that the candidate compound or molecule, if capable of acting as a transcriptional modulator of the gene encoding KCC2, KCC3 and/or KCC4, causes the polypeptide to be expressed and so produces a detectable signal, which can be assayed quantitatively and compared to an appropriate control. Candidate compounds or molecules of interest can include those

which increase or decrease, *i.e.*, modulate, transcription from the KCC2, KCC3 or KCC4 promoters. The reporter gene can encode a reporter known in the art, such as luciferase, or it can encode KCC2, KCC3 and/or KCC4.

In certain embodiments of the invention the polypeptide so produced is capable of complexing with an antibody or is capable of complexing with biotin. In this case the resulting complexes can be detected by methods known in the art. The detectable signal of this assay can also be provided by messenger RNA produced by transcription of said reporter gene. Exactly how the signal is produced and detected can vary and is not the subject of the present invention; rather, the present invention provides the nucleotide sequences and/or putative regulatory regions of KCC2, KCC3 and/or KCC4 for use in such an assay. The molecule to be tested in these methods can be a purified molecule, a homogenous sample, or a mixture of molecules or compounds. Further, in the method of the invention, the DNA in the cell can comprise more than one modulatable transcriptional regulatory sequence.

In accordance with the present invention there is also provided a rapid and high throughput screening method that relies on the methods described above. This screening method comprises separately contacting each of a plurality of substantially identical samples. In such a screening method the plurality of samples preferably comprises more than about 10^4 samples, or more preferably comprises more than about 5×10^4 samples.

F.3. Animal Models

In addition, animal-based systems can be used to identify compounds capable of modulating KCC2, KCC3 and/or KCC4 biological activity. Such animal models can be used for the identification of drugs, pharmaceuticals, therapies, and interventions that can be effective in modulating potassium-chloride cotransporter biological activity. For example, animal models can be exposed to a compound that is suspected of exhibiting an ability to modulate potassium-chloride cotransporter biological activity symptoms at a sufficient concentration and for a time sufficient to elicit such modulation of potassium-chloride cotransporter biological activity symptoms in the exposed animals. The response of the animals to the exposure can be monitored by assessing *in vivo*

potassium-chloride cotransporter expression levels and activity, or by testing biological samples from the animal. As in the methods described above, the mechanism by which a compound modulates KCC2, KCC3, KCC4, or other potassium-chloride cotransporter or ion transporter activity or achieves therapeutic effects can vary; the utility of the present invention does not depend on the precise mechanism by which an effect is achieved.

For example, an animal model of the present invention can comprises a mouse with targeted modification of the mouse KCC2, KCC3, and KCC4 genes. Mice strains with complete or partial functional inactivation of the KCC genes in all somatic cells are generated using standard techniques of site-specific recombination in murine embryonic stem cells. See Capecchi (1989) *Science* 244(4910):1288-92; Thomas & Capecchi (1990) *Nature* 346(6287):847-50; Delpire et al. (1999) *Nat Genet* 22(2):192-5. "Knockout" murine KCC2 mice have been prepared, and the resultant homozygous KCC2 -/- mice have a seizure disorder and increased perinatal mortality. The HCC2 knockout mice thus provide evidence that KCC2 is a drug target in epilepsy and further evidence that human KCC2 is a medically relevant gene.

Alternatives include the use of anti-sense or ribozyme KCC constructs, driven by a universal or tissue-specific promoter, to reduce levels of individual KCCs in somatic cells, thus achieving a "knock-down" of individual isoforms (Luyckx et al. (1999) *Proc Natl Acad Sci USA* 96(21):12174-12179). The invention also provides the generation of murine strains with conditional or inducible inactivation of individual or multiple KCC genes (Sauer (1998) *Methods* 14(4):381-392). For example, mice are created which lack expression of any KCCs in the renal proximal tubule, a known site of expression of KCC3 and KCC4, through the sequential mating of mice strains with lox-P-flanked KCC genes with a transgenic line expressing Cre-recombinase in the proximal tubule, using the promoter for the kidney androgen-regulated protein (Ding et al. (1997) *J Biol Chem* 272(44):28142-28148).

The present invention also provides mice strains with specific "knocked-in" modifications in the KCC2, KCC3, or KCC4 genes. This includes mice with genetically (Forlino et al. (1999) *J Biol Chem* 274(53):37923-37931) and

functionally (Kissel et al. (2000) *EMBO J* 19(6):1312-1326) relevant point mutations in the KCC genes, in addition to manipulations such as the insertion of disease-specific repeat expansions (White et al. (1997) *Nat Genet* 17(4):404-410).

An aspect of the invention encompasses any treatments that alter any aspect of potassium-chloride-cotransporter-mediated biological activity. Such compounds should be considered as candidates for human therapeutic intervention in accordance with the methods described herein below. Dosages of test agents can be determined by deriving dose-response curves, such as those disclosed in U.S. Patent No. 5,849,578, herein incorporated by reference.

G. Therapeutic Methods

As used herein, the terms "activity" and "biological activity" are meant to be synonymous and are meant to refer to any biological activity of, for example, a KCC2, KCC3 and/or KCC4 polypeptide. Representative biological activities of KCC3 and/or KCC4 comprise activity in the modulation of potassium-chloride cotransporter activity or other biological activity in accordance with the present invention.

With respect to the therapeutic methods of the present invention, a preferred subject is a vertebrate subject. A preferred vertebrate is warm-blooded; a preferred warm-blooded vertebrate is a mammal. A preferred mammal is a mouse or, most preferably, a human. As used herein and in the claims, the term "patient" is contemplated to include both human and animal patients. Thus, veterinary therapeutic uses are contemplated in accordance with the present invention.

Contemplated is the treatment of mammals such as humans, as well as those mammals of importance due to being endangered, such as Siberian tigers; of economical importance, such as animals raised on farms for consumption by humans; and/or animals of social importance to humans, such as animals kept as pets or in zoos. Examples of such animals include but are not limited to: carnivores such as cats and dogs; swine, including pigs, hogs, and wild boars; ruminants and/or ungulates such as cattle, oxen, sheep, giraffes, deer, goats, bison, and camels; and horses. Also contemplated is the

treatment of birds, including the treatment of those kinds of birds that are endangered and/or kept in zoos, as well as fowl, and more particularly domesticated fowl, *i.e.*, poultry, such as turkeys, chickens, ducks, geese, guinea fowl, and the like, as they are also of economical importance to humans. Thus, contemplated is the treatment of livestock, including, but not limited to, domesticated swine, ruminants, ungulates, horses, poultry, and the like.

G.1. Modulation of Potassium-chloride Cotransporter Biological Activity

In one embodiment, a therapeutic method according to the present invention comprises administering to a subject a substance that modulates, *i.e.*, inhibits or promotes potassium-chloride cotransporter biological activity. Such a substance can be identified according to any of the screening assays set forth above, either *in vitro* or *in vivo*. The method comprises treating a vertebrate subject suffering from a disorder associated with or mediated by potassium-chloride cotransporter biological activity by administering to the subject an effective amount of a substance identified according to a screening assay described above. By the term "modulating", it is contemplated that the substance can either promote or inhibit the biological activity of potassium-chloride cotransporter polypeptides, depending on the disorder to be treated, and can affect one or several of the potassium-chloride cotransporters, including KCC2, KCC3 and/or KCC4, as well as other isoforms of potassium-chloride cotransporters, ion transporters, or other unrelated genes or gene products. Therapeutic treatment can comprise the administration of antibodies against a chosen region of potassium-chloride cotransporters, the administration of a protein that enhances activity, or the administration of a protein that inhibits the transcription of the potassium-chloride cotransporter. Such administration can provide treatment of disorders which can be caused or exacerbated by potassium-chloride-cotransporter-mediated mechanisms, including but not limited to hypertension, epilepsy, sickle cell anemia, Bartter's syndrome, and Meniere's disease.

Insofar as a modulator of potassium-chloride-cotransporter activity can take the form of a polypeptide or of an anti-potassium-chloride-cotransporter monoclonal antibody or fragment thereof, it is to be appreciated that the

potency can vary, and therefore a "therapeutically effective" amount can vary. However, as shown by the present assay methods, one skilled in the art can readily assess the potency and efficacy of a candidate potassium-chloride cotransporter biological activity modulator of this invention and adjust the therapeutic regimen accordingly. A modulator of potassium-chloride-cotransporter biological activity can be evaluated by a variety of means including through the use of a responsive reporter, which drives expression of a reporter gene; interaction of potassium-chloride cotransporter polypeptides with a monoclonal antibody as described herein; and other assays known in the art and described herein.

The monoclonal antibodies or polypeptides of the invention can be administered parenterally by injection or by gradual infusion over time. Although the tissue to be treated can typically be accessed in the body by systemic administration and therefore most often treated by intravenous administration of therapeutic compositions, other tissues and delivery means are contemplated where there is a likelihood that the tissue targeted contains the target molecule and are known to those of skill in the art. The compositions are formulated in an appropriate manner and administered in a manner compatible with the dosage formulation.

G.2. Monoclonal Antibodies

The present invention describes, in one embodiment, potassium-chloride cotransporter modulators in the form of monoclonal antibodies which were elicited in response to KCC2, KCC3 and/or KCC4 but which can immunoreact with any potassium-chloride cotransporter polypeptide, or with a specific isoform of a potassium-chloride cotransporter polypeptide, and bind the potassium-chloride cotransporter polypeptide to modulate biological activity. The invention also describes cell lines that produce the antibodies, methods for producing the cell lines, and methods for producing the monoclonal antibodies.

The term "antibody" or "antibody molecule" refers collectively to a population of immunoglobulin molecules and/or immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain a paratope. A

paratope is the portion or portions of an antibody that is or are responsible for that antibody binding to an antigenic determinant, or epitope.

Representative antibodies for use in the present invention are intact immunoglobulin molecules, substantially intact immunoglobulin molecules, single chain immunoglobulins or antibodies, those portions of an immunoglobulin molecule that contain the paratope, including antibody fragments. Indeed, it is contemplated to be within the scope of the present invention that a monovalent modulator can optionally be used. Thus, the terms "modulate", "modulating", and "modulator" are intended to encompass such a mechanism.

The term "monoclonal antibody" refers to a population of antibody molecules that contain only one species of paratope and thus typically display a single binding affinity for any particular epitope with which it immunoreacts; a monoclonal antibody can have a plurality of antibody combining sites, each immunospecific for a different epitope, *e.g.*, a bispecific monoclonal antibody. Methods of producing a monoclonal antibody, a hybridoma cell, or a hybridoma cell culture are described above.

It is also possible to determine, without undue experimentation, if a monoclonal antibody has the same or equivalent specificity or immunoreaction characteristics as a monoclonal antibody of this invention by ascertaining whether the former prevents the latter from binding to a preselected target molecule. If the monoclonal antibody being tested competes with the monoclonal antibody of the invention, as shown by a decrease in binding by the monoclonal antibody of the invention in standard competition assays for binding to the target molecule when present in the solid phase, then it is likely that the two monoclonal antibodies bind to the same, or a closely related, epitope.

Still another way to determine whether a monoclonal antibody has the specificity of a monoclonal antibody of the invention is to pre-incubate the monoclonal antibody of the invention with the target molecule with which it is normally reactive, and then add the monoclonal antibody being tested to determine if the monoclonal antibody being tested is inhibited in its ability to bind the target molecule. If the monoclonal antibody being tested is inhibited

then, in all likelihood, it has the same, or functionally equivalent, epitopic specificity as the monoclonal antibody of the invention.

An additional way to determine whether a monoclonal antibody has the specificity of a monoclonal antibody of the invention is to determine the amino acid residue sequence of the CDR regions of the antibodies in question. "CDRs" (complementarity-determining regions) mean the three subregions of the light or heavy chain variable regions which have hypervariable sequences and form loop structures that are primarily responsible for making direct contact with antigen. Antibody molecules having identical, or functionally equivalent, amino acid residue sequences in their CDR regions have the same binding specificity. Methods for sequencing polypeptides are well known in the art. Further, other ways of determining whether antibodies have similar immunospecificities are known in the art and can be useful in practicing the present invention.

The immunospecificity of an antibody, its target molecule binding capacity, and the attendant affinity the antibody exhibits for the epitope are defined by the epitope with which the antibody immunoreacts. The epitope specificity is defined at least in part by the amino acid residue sequence of the variable region of the heavy chain of the immunoglobulin that comprises the antibody, and in part by the light-chain-variable-region amino acid residue sequence. Use of the terms "having the binding specificity of" or "having the binding preference of" indicates that equivalent monoclonal antibodies exhibit the same or similar immunoreaction (binding) characteristics and compete for binding to a preselected target molecule.

Humanized monoclonal antibodies offer particular advantages over monoclonal antibodies derived from other mammals, particularly insofar as they can be used therapeutically in humans. Specifically, human antibodies are not cleared from the circulation as rapidly as "foreign" antigens, and do not activate the immune system in the same manner as foreign antigens and foreign antibodies. Methods of preparing "humanized" antibodies are generally well known in the art, and can readily be applied to the antibodies of the present invention.

The use of a molecular cloning approach to generate antibodies, particularly monoclonal antibodies, and more particularly single chain monoclonal antibodies, is also contemplated. The production of single chain antibodies has been described in the art, see *e.g.*, U.S. Patent No. 5,260,203, the contents of which are herein incorporated by reference. For this approach, combinatorial immunoglobulin phagemid libraries are prepared from RNA isolated from the spleen of the immunized animal, and phagemids expressing appropriate antibodies are selected by panning on endothelial tissue. The advantages of this approach over conventional hybridoma techniques are that approximately 10^4 times as many antibodies can be produced and screened in a single round, and that new specificities are generated by hours and L chain combination in a single chain, which further increases the chance of finding appropriate antibodies. Thus, an antibody of the present invention, or a "derivative" of an antibody of the present invention, pertains to a single polypeptide chain binding molecule which has binding specificity and affinity substantially similar to the binding specificity and affinity of the light and heavy chain aggregate variable region of an antibody described herein.

G.3. Other Modulators

Given the disclosure of the potassium-chloride cotransporter activity in tissues herein, it is also contemplated that chemical compounds (*e.g.,* small molecule mimetics) can be used to modulate potassium-chloride cotransporter activity in tissues in accordance with the methods of the present invention. The identification of such compounds is facilitated by the description of screening assays directed to potassium-chloride cotransporter activity in tissues presented above.

For example, the initial functional data (Figures 24-25) indicate that agents that enhance protein phosphatase-1 can increase K^+- Cl^- cotransport activity. Derivatives of anion transport inhibitors such as bumetanide, furosemide, DIDS, and DIOA can have significant inhibitory potential for the K^+-Cl^- cotransporters. Finally, structure-function studies using the four KCCs, in addition to mutants and chimeras thereof, can yield important data crucial for the generation of KCC-specific and isoform-specific inhibitors.

G.4. Gene Therapy

Potassium-chloride cotransporter genes can be used for gene therapy in accordance with the present invention. Exemplary gene therapy methods, including liposomal transfection of nucleic acids into host cells, are described in U.S. Patent Nos. 5,279,833; 5,286,634; 5,399,346; 5,646,008; 5,651,964; 5,641,484; and 5,643,567, the contents of each of which are herein incorporated by reference.

Briefly, gene therapy directed toward modulation of potassium-chloride cotransporter levels, to thereby affect or modulate the biological activity of potassium-chloride cotransporter in a target cell is described. This modulation can affect cell growth and differentiation. In one embodiment, a therapeutic method of the present invention provides a process for modulation of potassium-chloride cotransporter levels comprising the steps of: (a) delivering to the cell an effective amount of a DNA molecule comprising a polynucleotide that encodes a polypeptide that modulates the biological activity of one or more than one potassium-chloride cotransporter; and (b) maintaining the cell under conditions sufficient for expression of said polypeptide.

In a preferred embodiment, the delivered polypeptide comprises the sequence of any of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and 112. Delivery can be accomplished by injecting the DNA molecule into the cell. Where the cell is in a subject, administering comprises the steps of: (a) providing a vehicle that contains the DNA molecule; and (b) administering the vehicle to the subject.

A vehicle is preferably a cell transformed or transfected with the DNA molecule or a transfected cell derived from such a transformed or transfected cell. An exemplary and preferred transformed or transfected cell is a lymphocyte or a tumor cell from the tumor being treated. Means for transforming or transfecting a cell with a DNA molecule of the present invention are set forth above.

Alternatively, the vehicle is a virus or an antibody that specifically infects or immunoreacts with an antigen of the target tissue or tumor. An advantage of a viral infection system is that it allows for a very high level of infection into the

appropriate recipient cell. Also, antibodies have been used to target and deliver DNA molecules.

It is also envisioned that this embodiment of the present invention can be practiced using alternative viral or phage vectors, including retroviral vectors and vaccinia viruses whose genome has been manipulated in alternative ways so as to render the virus non-pathogenic. Methods for creating such a viral mutation are set forth in detail in U.S. Patent No. 4,769,331, incorporated herein by reference.

G.5. Method of Modulating *In vivo* Potassium-Chloride Cotransporter Levels in the Treatment of Related Diseases and Disorders

A method for transcriptionally modulating in a multicellular organism the expression of a gene encoding a potassium-chloride cotransporter to modulate potassium-chloride cotransporter biological activity in a warm-blooded vertebrate subject is also contemplated in accordance with the present invention. This method comprises administering to the warm-blooded vertebrate subject a compound at a concentration effective to transcriptionally modulate expression of potassium-chloride cotransporter or cotransporters.

In accordance with the present invention, the contemplated compound can optionally comprise an antibody or polypeptide prepared as described above and which transcriptionally modulates expression of potassium-chloride cotransporters. Optionally, the antibody or polypeptide directly binds to DNA or RNA, or directly binds to a protein involved in transcription.

Particularly contemplated chemical entities (*e.g.,* small molecule mimetics) for use in accordance with the present invention do not naturally occur in any cell, whether of a multicellular or a unicellular organism. Even more particularly, the contemplated chemical entity is not a naturally occurring molecule, *e.g.,* it is a chemically synthesized entity. Optionally, the compound can bind a modulatable transcription sequence of the gene. For example, the compound can bind a promoter region upstream of a nucleic acid sequence encoding KCC2, KCC3 and/or KCC4 as well as other potassium-chloride cotransporters.

In the methods above, modulation of transcription results in either upregulation or downregulation of expression of the gene encoding the protein of interest, depending on the identity of the molecule which contacts the cell.

G.6. Antisense Oligonucleotide Therapy

It is also contemplated according to the present invention that expression of a potassium-chloride cotransporter can be modulated in a vertebrate subject through the administration of an antisense oligonucleotide derived from a nucleic acid molecule encoding a potassium-chloride cotransporter, such as those described in any of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and 112. Therapeutic methods utilizing antisense oligonucleotides have been described in the art, for example, in U.S. Patent Nos. 5,627,158 and 5,734,033, the contents of each of which are herein incorporated by reference.

G.7. Dosages

As used herein, an "effective" dose refers to one that is administered in doses tailored to each individual patient manifesting symptoms of K^+-Cl^- cotransport malfunction sufficient to cause an improvement therein. After review of the disclosure herein of the present invention, one of ordinary skill in the art can tailor the dosages to an individual patient, taking into account the particular formulation and method of administration to be used with the composition as well as patient height, weight, severity of symptoms, and stage of the disorder to be treated.

An effective dose and a therapeutically effective dose are generally synonymous. However, compounds can be administered to patients having reduced symptoms or even administered to patients as a preventative measure. Hence, the composition can be effective in therapeutic treatment even in the absence of symptoms of the disorder.

A unit dose can be administered, for example, 1 to 4 times per day. Most preferably, the unit dose is administered twice a day (BID). The dose depends on the route of administration and the formulation of a composition containing the compound or compounds. Further, it will be appreciated by one of ordinary skill in the art after receiving the disclosure of the present invention that it can be necessary to make routine adjustments or variations to the

dosage depending on the combination of agents employed, on the age and weight of the patient, and on the severity of the condition to be treated.

Such adjustments or variations, as well as evaluation of when and how to make such adjustments or variations, are well known to those of ordinary skill in the art of medicine. Evaluation parameters and techniques can vary with the patient and the severity of the disease. Particularly useful evaluative techniques are disclosed in the Examples.

G.7.1. Gene Therapy Vector Construct Dosing

Maximally tolerated dose (MTD) of vector construct when administered directly into the affected tissue is determined. Primary endpoints are: 1) the rate of transduction in abnormal and/or normal cells, 2) the presence and stability of this vector in the systemic circulation and in affected cells, and 3) the nature of the systemic (fever, myalgias) and local (infections, pain) toxicities induced by the vector. A secondary endpoint is the clinical efficacy of the vector construct.

For example, a 4 ml serum-free volume of viral (*e.g.,* adenoviral, retroviral, *etc.*) vector construct (containing up to 5 $X10^7$ viral particles in AIM V media) is administered daily per session. During each session, 1 ml of medium containing the appropriate titer of vector construct is injected into 4 regions of the affected tissue for a total of 4 ml per session in a clinical examination room. This is repeated daily for 4 days (4 sessions). This 16 ml total inoculum volume over 4 days is proportionally well below the one safely tolerated by nude mice (0.5 ml/20 g body weight).

Patient evaluation includes history and physical examination prior to initiation of therapy and daily during the 4 day period of vector construct injection. Toxicity grading is done using the ECOG Common Toxicity Criteria. CBC, SMA-20, urinalysis, and conventional studies are performed daily during this period.

G.7.2. Dose escalation and MTD

Patients are treated with 3 x 10^6 viral particles x 4. Once they have all recovered from all grade 2 or less toxicities (except alopecia), and as long as grade 3-4 toxicity is not encountered, a subsequent dose level is initiated in

patients. As one grade 3 or 4 toxicity occurs at a given dose level, a minimum of 6 patients are enrolled at that level. As only 1 of 6 patients has grade 3 or 4 toxicity, dose escalation continues. The MTD of vector construct is defined as the dose where 2 of 6 patients experience grade 3 or 4 toxicity. If 2 of 3, or if 3 of 6 patients experience grade 3 or 4 toxicity, the MTD is defined as the immediately lower dose level.

The following escalation schema is followed: 1) level 1, 3 x 10^6 viral particles; 2) level 2, 1 x 10^7; 3) level 3, 3 x 10^7; 4) level 4, 5 X 10^7. Patients with measurable disease are evaluated for a clinical response to vector construct. Histology and local symptoms are followed. NE clearance, tyramine administration and other standard tests such as are disclosed in the Examples are employed.

G.8. Formulation of Therapeutic Compositions

The potassium-chloride-cotransporter biological activity modulating substances, gene therapy vectors, and substances that inhibit or promote expression of a potassium-chloride cotransporter encoding nucleic acid segment described above are adapted for administration as a pharmaceutical compositions as described above. Additional formulation and dose preparation techniques have been described in the art, see for example, those described in U.S. Patent No. 5,326,902 issued to Seipp et al. on July 5, 1994, U.S. Patent No. 5,234,933 issued to Marnett et al. on August 10, 1993, and International Publication Number WO 93/25521 of Johnson et al. published December 23, 1993, the entire contents of each of which are herein incorporated by reference.

For the purposes described above, the identified substances can normally be administered systemically or partially, usually by oral or parenteral administration. The doses to be administered are determined depending upon age, body weight, symptom, the desired therapeutic effect, the route of administration, and the duration of the treatment, *etc.*; one of skill in the art of therapeutic treatment will recognize appropriate procedures and techniques for determining the appropriate dosage regimen for effective therapy. Various compositions and forms of administration are contemplated and are generally known in the art. Other compositions for administration include liquids for

external use, and endermic linaments (ointment, *etc.*), suppositories and pessaries that comprise one or more of the active substance(s) and can be prepared by known methods.

Thus, the present invention provides pharmaceutical compositions comprising a polypeptide, polynucleotide, or molecule or compound of the present invention and a physiologically acceptable carrier. More preferably, a pharmaceutical composition comprises a compound discovered via the screening methods described herein below.

A composition of the present invention is typically administered parenterally in dosage unit formulations containing standard, well-known nontoxic physiologically acceptable carriers, adjuvants, and vehicles as desired. The term "parenteral" as used herein includes intravenous, intra-muscular, intra-arterial injection, or infusion techniques.

Injectable preparations, for example sterile injectable aqueous or oleaginous suspensions, are formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol.

Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

Preferred carriers include neutral saline solutions buffered with phosphate, lactate, Tris, and the like. Of course, one purifies the vector sufficiently to render it essentially free of undesirable contaminants, such as defective interfering adenovirus particles or endotoxins and other pyrogens such that it does not cause any untoward reactions in the individual receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation.

A transfected cell can also serve as a carrier. By way of example, a liver cell can be removed from an organism, transfected with a polynucleotide of the present invention using methods set forth above and then the transfected cell returned to the organism (*e.g.,* injected intra-vascularly).

EXAMPLES

The following Examples have been included to illustrate modes of the invention. Certain aspects of the following Examples are described in terms of techniques and procedures found or contemplated by the present inventors to work well in the practice of the invention. These Examples are exemplified through the use of standard laboratory practices of the inventors. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following Examples are intended to be exemplary only and that numerous changes, modifications and alterations can be employed without departing from the spirit and scope of the invention.

Example 1

Cloning of mKCC4 cDNA

A BLAST (basic alignment and search tool) search of the EST data base revealed a number of mouse ESTs that were homologous to rat KCC1 and KCC2. The cDNAs corresponding to four of these ESTs (IMAGE clones 568084, 633794, 313521, and 698105) were obtained from the IMAGE consortium (Research Genetics, Genome Systems and/or the American Type Culture Collection) and sequenced. These ESTs contained partial open reading frames homologous to various segments of KCC1 and KCC2 (Figure 1). The 3' end of the open reading frame and the entire 3'-UTR were identified in a fifth IMAGE cDNA (clone 1314678).

The tissue distribution of mKCC4 was then assessed by RT-PCR, and widespread expression of mKCC4 was detected, with particularly abundant transcripts in kidney and heart. Using a primer pair spanning the gap between the IMAGE clones 313521 and 568084, a 1.2-kb fragment was amplified from mouse strain C57BL/6J kidneys and subcloned into the EcoR V site of the vector pBluescript by blunt-end ligation. PCR conditions for these and other

gene-specific primers were optimized using Taq 2000 and the Opti-Prime buffer system (available from Stratagene of La Jolla, California). The following amplification protocol was followed, unless specified otherwise: 30 cycles of denaturation (92°C, 2 minutes), annealing (54°C, 1 minute), and extension (72°C, 1 minute), followed by a final extension step (72°C, 8 minutes). The extreme 5'-end of mKCC4 was cloned from BALB/c mouse kidney 5'-RACE template (Clontech of Palo Alto, California), using two antisense primers and the AP1 adaptor primer/S3 primer (Clontech of Palo Alto, California). This PCR utilized AmpliTaq-Gold DNA polymerase (Perkin-Elmer Corp. of Boston, Massachusetts) and a hot-start amplification protocol, consisting of a 9-minute enzyme activation step at 94°C, followed by 35 cycles of 94°C for 1 minute, 68°C for 2.5 minutes, and a final 10-minute extension at 72°C.

Example 2

Cloning of human KCC4 cDNA

A human KCC EST clone (TIGR clone 150738) was obtained from the ATCC. DNA sequencing revealed that this cDNA is derived from the human ortholog of mKCC4. The polypeptide encoded by another human 5'-EST (GenBank Accession No. F12342) exhibited strong amino acid homology with the amino terminus of the other KCCs. This EST overlaps with a large number of ESTs from the 3'-UTR of hKCC4, including the IMAGE clones 22250 and 51311 (Figure 1). The gap between these cDNAs and the TIGR clone 150738 (Figure 1) was bridged by RT-PCR with a primer pair, using a human kidney template (Clontech of Palo Alto, California). The PCR products for this reaction were subcloned into pBluescript (Stratagene of La Jolla, California). Finally, the 5'-end of hKCC4 was cloned by sequential RT-PCR of human kidney.

Example 3

Cloning of human KCC3a cDNA

Sequence analysis of another human EST cDNA (TIGR clone 150620) indicated the existence of a fourth KCC, hKCC3A. Northern blot analysis revealed significant expression in muscle, and a single 5'-RACE cDNA was cloned from a human muscle template (Clontech of Palo Alto, California). This 5'-RACE PCR used Advantage polymerase mix (Clontech of Palo Alto,

California) and a hot-start protocol consisting of the following: 94°C for 1 minute, followed by 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 68°C for 3 minutes. Screening of a random-primed human muscle cDNA library (Clontech of Palo Alto, California) with a probe encompassing nucleotides 741 to 871 of hKCC3 yielded a single cDNA that extended 5' of the start codon. Finally, two overlapping PCR fragments were amplified from human brain template (Clontech of Palo Alto, California), and subcloned into pCR2.1 by TA cloning (Invitrogen of Carlsbad, California).

The human KCC3a cDNA shares identity with another cDNA, human KCC3b, reported by Hiki et al. (1999) *J Biol Chem* 274(15):10661-10667, but differs in the first 40 amino acids of the amino terminus of the predicted transporter protein. Genomic characterization of mouse and human KCC3 indicates that this variation is generated by transcriptional initiation at two separate promoters 5 of two separate first coding exons, which have been denoted exon 1a and exon 1b. A partial cDNA corresponding to this human KCC3b isoform was cloned by RT-PCR, using a human KCC3b sense primer derived from the sequence of reported by Hiki et al. (1999) *J Biol Chem* 274(15):10661-10667 with an anti-sense primer from within sequence common to both variants of KCC3. RT-PCR of human, mouse and *Xenopus* tissue (see Figure 25B) also reveals conserved alternative splicing of exon 2, a 45 nucleotide exon whose alternative exclusion deletes 15 amino acids from the amino terminus while preserving the rest of the open reading frame.

Example 4

Cloning of mouse KCC3a and KCC3b cDNAs

The sequence data and cDNA clones for human KCC3 were used to determine the full-length cDNA sequences for mouse orthologues of KCC3. First, coding sequence oligonucleotide primer pairs from the human KCC3a and KCC3b were used to "walk" along the mouse KCC3a and KCC3b cDNA sequences by sequential RT-PCR of mouse tissue RNA. The entire mouse 3'-UTR was identified by sequencing of a mouse EST cDNA (IMAGE clone 1290431) that exhibited sequence homology to the human KCC3 3'-UTR. The gap between this 3' non-coding sequence and the known 3' coding sequence

was bridged by RT-PCR. To clone the two major 5' ends, which have been denoted KCC3a and KCC3b, primers designed according to human KCC3 sequences were used to amplify KCC3 partial clones from mouse RNA. The extreme 5' end of the mouse KCC3a and KCC3b sequences were then determined by sequencing of mouse genomic clones. The resultant full-length mouse KCC3a and KCC3b cDNAs are 6120 and 6052 nucleotides in length, respectively.

Example 5

Cloning of Human KCC2 cDNA

The 5' end of human KCC2 was cloned by RT-PCR of reverse-transcribed human brain poly-A^+ RNA using a human KCC2 anti-sense primer and a rat KCC2 sense primer. Two separate cDNAs, each extending from nucleotides 1-1650 of hKCC2, were cloned from two separate PCR reactions and subcloned into pCR2.1 (Invitrogen of Carlsbad, California). Overlapping sequence for the 3' end of hKCC2 was obtained from two 3' EST cDNAs, IMAGE clones 363600 (nucleotides 1370-3327 of hKCC2) and 362310 (nucleotides 3320-5907). The two IMAGE clones overlap at a common Not I site (3322 of hKCC2).

Example 6

Sequence Analysis of KCC2, KCC3, and KCC4 cDNAs

All cDNA clones were sequenced on both strands using fluorescent dye terminator chemistry (Applied Biosystems of Foster City, California). For cDNA sequence derived exclusively from PCR, at least two cDNAs from two separate PCR reactions were sequenced. Analyses of the nucleotide and amino acid sequences were performed using the GENEWORKS™ 2.5 and MACVECTOR™ 6.5 software packages (Oxford Molecular, Inc. of Campbell, California). Alignments and other analyses also made use of the computer programs BLAST (http://www.ncbi.nlm.nih.gov/blast), DNASTAR (DNASTAR, Inc. of Madison, Wisconsin), and SMART (simple modular architecture research tool) (Schultz et al. (1998) *Proc Natl Acad Sci USA* 95:5857-5864; Schultz et al.

(2000) *Nuc Acids Res* 28:231-234; available from EMBL of Heidelberg, Germany).

The complete mouse and human KCC4 cDNAs are 5132 and 5230 nucleotides long, respectively, which is close in size to the KCC3 transcripts seen on Northern blots. Both cDNAs contain open reading frames of 3248 nucleotides that are 85% identical. The predicted proteins consist of 1083 amino acids and exhibit 91% identity. The KCC4 3-UTRs are only 34% identical, close to the lower limit of conservation between mouse and human orthologs (Makalowski & Boguski (1998) *Proc Natl Acad Sci USA* 95:9407B9412).

The hKCC3a cDNA sequence is 4237 nucleotides in length, with a 5'-UTR of 165 base pairs and a 3'-UTR of 622 base pairs. There are three in-frame start codons between nucleotides 165 and 195 of the hKCC3a cDNA, at which translational initiation would result in proteins of 1150, 1141, or 1135 amino acids in length. However, homology to KCC1 and KCC4 begins before the third methionine, which is thus an unlikely translational start site. Comparison with the mouse KCC3a sequence indicates significant conservation of the first nine codons, which also contain a PKC site, and hence translation likely occurs at the first start codon. At least two KCC3 transcripts of 6B7 kb are detected by Northern blot analysis, consistent with alternative splicing. In comparison with other KCC3 cDNAs (Figure 1), the single 5'-RACE cDNA contained a deletion of nucleotides 708B854, encoding TM1 and TM2 in the predicted KCC3 protein. The deleted region corresponds precisely to exon 4 of hKCC1, and hence at least part of the heterogeneity in KCC3 transcripts is the result of the alternative splicing of coding exons. RT-PCR experiments indicate that exon 2 of both human, mouse and *xenopus* KCC3a is omitted in a significant fraction of transcripts, corresponding to the deletion of 15 amino acids from the predicted amino terminus.

The four KCC proteins are 65B75% identical. Sequence alignments indicate that hKCC4 shares 69% identity with rat KCC2, 65% identity with hKCC1, and 66% identity with hKCC4. The hKCC3a protein shares 71% identity with hKCC1, 66% identity with rat KCC2, and 66% identity with hKCC4.

Human KCC3b is marginally more similar to hKCC1 (75% identity) than is hKCC3a. The mouse and human KCC3a and KCC3b orthologues are 98% identical. Human KCC2 is in turn 99% identical to the rat KCC2 protein. The identity between the KCCs and other cation-chloride cotransporters is about 27-33%.

A phylogenetic tree indicates that the mammalian cation-chloride cotransporters fall into two groups, one composed of the Na^{+}-K^{+}-$2Cl^{-}$ cotransporters and the Na^{+}-Cl^{-} cotransporter, and the other encompassing the four K^{+}-Cl^{-} cotransporters. As indicated by direct sequence alignments, the four KCCs form two subgroups, one group comprising KCC1 and KCC4, and the other group comprising KCC3 and KCC2.

The seven mammalian cation-chloride cotransporters share a predicted membrane topology. A central core of 12 TM domains is flanked by hydrophilic amino- and carboxyl-terminal domains that have a cytoplasmic orientation (Mount et al. (1998) *J Exp Biol* 201:2091B2102). The major structural difference between the KCCs and the Na^{+}1-linked cotransporters is the position of a large glycosylated extracellular loop, which is predicted to occur between TM5 and TM6 in the KCCs and between TM7 and TM8 in the Na^{+1}-K^{+1}-$2Cl^{-2}$ cotransporters and the Na^{+1}-Cl^{-2} cotransporter (Mount et al. (1998) *J Exp Biol* 201:2091B2102). Homology is most marked in the TM domains, the intracellular loops, and the cytoplasmic carboxyl terminus.

A comparison of the four KCCs reveals a number of intriguing differences. KCC3a is the longest of the four because of an extension of 60 amino acids at the extreme amino-terminal end. Although highly conserved, none of the TM domains in the four KCCs are completely identical. Within the cytoplasmic domains, the four KCCs differ significantly in the distribution of consensus phosphorylation sites for tyrosine kinases, protein kinase A, and protein kinase C (PKC). A carboxyl-terminal tyrosine phosphorylation site in KCC2 (Tyr 1087) is conserved in mouse and human KCC4 (Tyr 1054). The hKCC3a sequence predicts a total of 11 PKC sites, 7 contained within the first 90 amino acids. KCC3a has two potential protein kinase A sites, one of which (Ser 939) is a predicted substrate for both protein kinases A and C. The KCC4

sequences predict fewer PKC sites, of which only two are conserved in both mouse and human (Thr 814 and Ser 1006).

Based on the primary structure of the KCCs, disclosed herein for KCC1, KCC3, and KCC4, and the kinetic characterization of NKCC1 chimeras (Isenring & Forbush (1997) *J Biol Chem* 272:24556-24562; Isenring et al. (1998a) *Proc Natl Acad Sci USA* 95:7179-7184; Isenring et al. (1998b) *J Biol Chem* 273:11295-11301; Isenring et al (1998c) *J Gen Physiol* 112:549-558), the four KCCs were proposed to differ in transport features due to variation within the central core of the transmembrane domains. In particular, TM2 is likely to control cation affinity, and TM4 and TM7 are likely to confer anion affinity. Such properties were tested by heterologous expression of KCCs in *Xenopus* oocytes as described further in Examples 11-15 herein below.

Example 7

Chromosomal Localization and Genomic Structure of hKCC2, hKCC3 and hKCC4

Chromosomal assignments for the human KCC3 and KCC4 genes were made using a PCR-based screening approach with the National Institute of General Medical Sciences (NIGMS) human/rodent somatic cell hybrid mapping panel 1. The primers used for hKCC4 mapping amplify a 377-base pair segment of the 3' noncoding region from genomic DNA, and the hKCC3 mapping primers amplify a 561-base pair segment of the 3' noncoding sequence. PCR reactions using DNA from the NIGMS panel were scored for the presence or absence of the appropriately sized product using agarose gel electrophoresis. The chromosomal localization of hKCC4 was verified by sequencing the chromosome 5 genomic clone pMS621 (set forth as SEQ ID NO:111). Fine mapping of hKCC3 was performed by PCR using radiation hybrid analysis with the Stanford G3 panel (Research Genetics of Boston, Massachusetts). Reaction products generated by PCR were alkali-denatured, applied to a nylon membrane using a dot-blot apparatus, and subjected to Southern blotting with a ^{32}P-labeled internal oligonucleotide probe. Results were analyzed by querying the Stanford radiation hybrid map (http://www-shgc.stanford.edu/RH/).

Chromosomal assignments for human KCC3 and KCC4 were defined using a PCR-based somatic cell hybrid mapping strategy. The genes for KCC3 and KCC4 were assigned to chromosomes 15 and 5, respectively. Fine mapping by radiation hybrid analysis places the hKCC3 gene on chromosome 15q14 between the markers D15S1040 and D15S118. Further localization of KCC4 was facilitated by the finding that D5S110 (Armour et al. (1996) *Ann Hum Genet* 60:11B20), a chromosome 5 VNTR (variable number of tandem repeats) mini-satellite marker, is contained within the gene. The corresponding genomic subclone, pMS621, was sequenced to verify that it contained hKCC3 exons just 5' of the VNTR. The exon/intron boundaries in the hKCC1 gene (Holtzman et al. (1998) *Am J Physiol* 275:F550BF564) are conserved in this portion of the hKCC4 gene, and the exons in pMS621 correspond to exons 14B17 of hKCC1. The chromosome 5 summary map generated by the Wessex Human Genetics Institute (http://cedar.genetics.soton.ac.uk/pub/chrom5/map.html) indicates that D5S110 is on chromosome 5p15.3, between D5S678 and the telomere.

The chromosomal localization of hKCC4 has been independently verified using the STS (sequence tag site) database. Thus the STS stSG1490 maps to chromosomal region 5p15, between the chromosome 5 markers D5S678 and D5S417. See Gene Map-99 URLs http://www.ncbi.nlm.nih.gov/genemap99/loc.cgi? ID=11342 and http://www.ncbi.nlm.nih.gov/genemap99/map.cgi? MAP=GB4&BIN=164&MARK=stSG1490.

The chromosomal localization of hKCC2 was also determined using the STS database. The STS WI-9597/stSG2530 is identical to nucleotides 5724-5896 of hKCC2. This STS maps to chromosomal region 20q13, between D20S836 and D20S888. See Gene Map-99 URLs http://www.ncbi.nlm.nih.gov/genemap99/loc.cgi? ID=35899 and http://www.ncbi.nlm.nih.gov/genemap99/map.cgi? MAP=GB4&BIN=583&MARK=stSG2530.

Genomic structure of hKCC2, hKCC3, and hKCC4 has been determined from the analysis of human genomic BAC (bacterial artificial chromosome) clones. These clones were identified by PCR-based screening of a human CITB BAC pool from Research Genetics (Boston, Massachusetts); in the case of one hKCC3 BAC, the clone was identified through a BLAST search of the TIGR and Washington University (St. Louis, Missouri) BACend databases.

The entire hKCC2 gene, at least encompassing all 24 coding exons, is found on the CITB clones 24H13 (exons 2-24) and 90C12 (exons 1-10). The entire hKCC3 gene, including at least 1.0 kb of promoter sequence 5 of exon 1a, is on the BAC clones R-122P18 (GenBank Accession No. AQ345102) and the CITB clone 278L3. Exons 2-24 of hKCC4 are contained within the CITB clone 330M20.

Intron-exon boundaries for the individual KCC exons were determined by direct sequencing of the BAC clones, using a modified chain termination protocol (BIGDYE™ available from PerkinElmer, Inc. of Boston, Massachusetts). In the case of hKCC2, this direct sequencing was not successful, and individual introns were amplified by PCR and long-range PCR, followed by subcloning into pCR2.1 (Clontech of Palo Alto, California); these subclones were then sequenced to determine intron-exon boundaries.

Figures 2A and 2B show that the four human KCC genes have all been localized to different chromosomes. The genomic structure of human KCC2, KCC3, and KCC4 has been determined by the characterization of human genomic BAC (bacterial artificial chromosome) clones. Intron-exon boundary sequence is known for all of hKCC3, and most of hKCC2 and hKCC4. Intron sizes have been mapped by PCR analysis of the relevant BAC clones, using primer pairs in adjacent exons to amplify each intron.

The genomic structure of hKCC3 is shown in Figure 2A. KCC3 has two separate first coding exons, denoted 1a and 1b. 5'-RACE PCR of mouse kidney has mapped the transcriptional start site of mKCC3b just 5' of the start codon in exon 1b, hence the two isoforms are generated by transcriptional initiation at two separate promoters. Human exon 1a has a 5-flanking CpG island (GenBank/EMBL Accession No. Z63283), previously identified by Cross et al. (1994) *Nat Genet* 6(3):236-44. The sequence for the putative mouse 1a and 1b promoters is included in SEQ ID NOs:18-19 respectively, and that of human exon 1a is included in SEQ ID NO:17.

Genomic structure of exons 2-24 of KCC4 is shown in Figure 2B. The polymorphic VNTR (variable number of tandem repeat) marker D5S110 (Armour et al. (1996) *Ann Hum Genet* 60(Pt 1):11-20), contained in the

genomic subclone pMS621, is found in the intron between exons 17 and 18 of hKCC4.

Example 8

Identification of a NSRE in KCC2

The mouse KCC2 gene contains a neuronal restrictive silencing element (NSRE) in intron 1, just 3′ of exon 1 (Karadesh & Delpire (2001) *J Neurophysiol* 85:995-997) (Figure 3). As disclosed herein, this element binds nuclear proteins from a murine neural progenitor cell line using a standard electromobility shift assay (Figures 4A and 4B). Given the function of neuronal restrictive silencing factor (NRSF) in repressing the expression of neuronal-specific genes in non-neuronal tissues (Schoenherr et al. (1996) *Proc Natl Acad Sci USA* 93:9881-9886), the expression of a KCC2 promoter construct containing the NRSE was tested in a non-neuronal cell line. Briefly, nuclear protein (15 μg) isolated from C17 neural progenitor cells was incubated with ^{32}P-end-labeled NSRE oligonucleotide for 15 minutes at room temperature. The reactions were resolved on a 4% acrylamide gel and visualized by autoradiographic exposure. Luciferase expression directed by a KCC2-NRSE construct in the PGL3 vector was reduced when compared to levels of luciferase expression directed by a promoter without the NRSE element in the PGL3 vector (Figure 5). The promoter constructs used comprised the mKCC2 promoter (SEQ ID NO:131), with or without the NSRE.

Example 9

Cloning of *Xenopus* KCC

Sequence of the last 2290 nucleotides of a *Xenopus* KCC (SEQ ID NO:112) was obtained from overlapping RT-PCR cDNA (cloned using primers derived from the EST PBX0118E03) and EST (IMAGE clone 3399678) clones. The sequence of the C-terminal 358 amino acids of "xKCC" (SEQ ID NO:113) reveals 76% identity with hKCC3. A tissue survey by RT-PCR indicates that this transcript is widely expressed, including within oocytes. The expression of a highly homologous KCC in oocytes validates these cells as a model expression system for the mammalian KCCs.

Example 10

In vitro Translation of mKCC4 Protein

One (1.0) mg of the full-length mKCC4 cDNA (SEQ ID NO:13) was translated *in vitro* using [^{35}S]methionine and T7 RNA polymerase-coupled rabbit reticulocyte lysate (TNT™ T7 RNA polymerase available from Promega of Madison, Wisconsin), both with and without pancreatic microsomes, for 90 minutes at 30°C. Protein was resolved by 7% SDS-polyacrylamide gel electrophoresis followed by autoradiography.

KCC1, KCC2, and other members of the cation-chloride cotransporter gene family are known to be glycoproteins, and the four KCC sequences contain three identical N-linked glycosylation sites in the otherwise poorly conserved extracellular loops. The *in vitro* translation of mKCC4 results in a protein with an apparent molecular mass of 115 kDa, slightly lower than the predicted core weight of 119 kDa. The addition of canine pancreatic microsomes results in the appearance of an additional band of higher molecular mass, which is consistent with *in vitro* glycosylation. Western blot analysis with KCC3- and KCC4-specific antibodies reveals that the native proteins are 40-60 kDa greater in mass than the predicted core proteins, consistent with glycosylation of the native proteins.

Example 11

Heterologous Expression of mKCC4 and hKCC3a in *X. laevis* Oocytes

Differences in KCC transmembrane domains were predicted to confer differences in K^+-Cl^- cotransport. Table 3 summarizes the differences among the KCCs in ion affinity, diuretic senstivity, and anion selectivity, as described further in Examples 14-15 herein below.

Table 3. Summary of KCC Properties

KCC	Potassium K_m (mM)	Chloride K_m (mM)	Furosemide K 0.5	Anion Series
KCC1	25.5	17.2	180 μM	$Cl^- > \underline{SCN^-} > Br^- > PO_4^- > I$
KCC2	9.3	6.3	90 μM	$Cl^- > Br^- > PO_4^- = I^- = SCN^-$
KCC3	51.9	14.2	180 μM	$\underline{Br^-} > Cl^- > PO_4^- = I^- > SCN^-$
KCC4	17.5	15.3	900 μM	$Cl^- > Br^- > PO_4^- = I^- > SCN^-$
xKCC	27.7	15.4	200 μM	$Cl^- = PO_4^- = Br^- > I^- > SCN^-$

Oocytes were surgically collected from anesthetized female *Xenopus* adults under 0.17% tricaine and incubated for 1 hour with vigorous shaking in frog Ringer ND96 (96 mM sodium chloride, 2 mM potassium chloride, 1.8 mM calcium chloride, 1.0 mM magnesium chloride, and 5mM Hepes/Tris, pH 7.4) in the presence of 2 mg/ml collagenase B. Oocytes were then washed four times in ND96, manually defolliculated, and incubated overnight in ND96 at 18°C. On the next day, defolliculated stage V-VI oocytes were injected with 50 nl of water or a solution containing cRNA at a concentration of 0.25-0.5 μg/μl (25 ng/oocyte). Oocytes were incubated at 17-18°C in ND96 (96 mM sodium chloride, 2 mM potassium chloride, 1.8 mM calcium chloride, 1.0 mM magnesium chloride, and 5mM Hepes/Tris, pH 7.4), supplemented with 2.5 mM sodium pyruvate and 5 mg/100 ml gentamicin, for 3-4 days. The incubation medium was changed every 24 hours. On the day of the experiment, oocytes were switched to Cl^--free ND96 (96 mM Na^+ Na^+ isethionate, 2 mM K^+ gluconate, 6 mM Ca^{2+} gluconate, 1 mM Mg^{2+} gluconate, 5 mM Hepes, 2.5 mM sodium pyruvate, 5 mg/100ml gentamicin, pH 7.4) for 2 hours prior to the uptake assay.

For functional expression and *in vitro* translation, a full-length mKCC4 cDNA was used as a standard in the *Xenopus* expression vector pGEMHE (Liman et al. (1992) *Neuron* 9:861-871). The resulting construct contains nucleotides 55-3812 of mKCC4 (SEQ ID NO:13). For functional comparison with mKCC4, a full-length rabbit KCC1 cDNA was subcloned into pGEMHE. A full-length hKCC3a construct has also been generated for functional expression, and comparative data for rabKCC1, hKCC3a, and mKCC4. To prepare a template for cRNA, the rabbit KCC1 and mKCC3 cDNAs were

linearized at the 3'-end using Nhe I, and cRNA was transcribed *in vitro* using the T7 RNA polymerase and the mMESSAGE mMACHINE kit (Ambion, Inc. of Austin, Texas). Transcription product integrity was confirmed on agarose gels, and concentration was determined by absorbance reading at 260 nm (DU 640 available from Beckman of Fullerton, California).

Example 12

Assessment of K^+-Cl^- Cotransport

K^+-Cl^- cotransport was assessed by measuring tracer $^{86}Rb^+$ uptake (NEN® Life Science Products of Boston, Massachusetts) in experimental groups of at least 15 oocytes. Since both KCC4 and KCC1 display minimal activity under isotonic conditions $^{86}Rb^+$ uptake was generally assessed in oocytes swollen by a 30-minute incubation period in a hypotonic K^+ and Cl^--free medium (50 mM N-methyl-D-glucamine (NMDG) gluconate, 4.6 mM Ca^{2+} gluconate, 1.0 mM Mg^{2+} gluconate, 5 mM Hepes, pH 7.4) with 1 mM ouabain, followed by a 60-minute uptake period in a hypotonic Na^+-free medium with variable K^+-Cl^- content. K^+ and Cl^- concentrations were varied separately using combinations of KCl, NMDG chloride, potassium gluconate, and NMDG gluconate, for a maximal total concentration of 50 mM; an uptake solution with 50 mM K^+-Cl^- did not contain NMDG chloride, potassium gluconate, or NMDG gluconate, for example. All uptake solutions also contained 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.4, and were supplemented with 1 mM ouabain and 5.0 mCi/ml $^{86}Rb^+$. Isotonic conditions were generated by supplementing the same solutions with 3.5 g/100 ml sucrose to reach isosmolar conditions for oocytes (~210 mOsm/kg). Ouabain was added to prevent $^{86}Rb^+$ uptake via the Na^+-K^+-ATPase. The absence of extracellular Na^+ and the hypotonicity of the uptake medium prevented $^{86}Rb^+$ uptake via the endogenous Na^+-K^+-$2Cl^-$ cotransporter that is present in oocytes (Gamba et al. (1994) *J Biol Chem* 269:17713-17722).

All uptake assays were performed at 32°C. At the end of the uptake period, oocytes were washed five times in ice-cold uptake solution without isotope to remove extracellular fluid tracer. Oocytes were dissolved in 10% SDS, and tracer activity was determined for each oocyte by β-scintillation

counting.

To determine the ion transport kinetics of KCC4 and KCC1, $^{86}Rb^+$ uptake experiments were performed in variable concentrations of K^+ and Cl^-. The sensitivity for several inhibitors was assessed by exposing groups of oocytes to the inhibitors at concentrations varying from 20 mM to 2 mM. For these experiments, the desired concentration of the inhibitor was present during both the incubation and uptake periods, except when noted.

In isotonic conditions, no differences were observed among KCC4, KCC1, and water-injected oocytes. When uptake experiments were performed under hypotonic conditions, microinjection of KCC4 and KCC1 cRNAs resulted in significant K^+-Cl^- transport activity, as compared with control ooctyes that were injected with water. Figure 6 summarizes five experiments in which oocyes from difference frogs were injected with water or KCC4 or KCC1 cRNA, followed by $^{86}Rb^+$ uptake assay using a hypotonic uptake solution containing 10 mM and 50 mM of extracellular K^+ and Cl^-, respectively. In control oocytes, $^{86}Rb^+$ uptake was 588 ± 91 pmol X oocyte^{-1} X hour $^{-1}$ in the presence of Cl^- and 147 ± 23 pmol X oocyte^{-1} X hour $^{-1}$ in the absence of Cl^-, indicating the presence of an endogenous K^+-Cl^- cotransporter. Microinjection of KCC4 cRNA resulted in an increased $^{86}Rb^+$ uptake to 24,457 ± 3,476 pmol X oocyte^{-1} X hour^{-1}. This $^{86}Rb^+$ uptake was Cl^- -dependent, in that uptake in KCC4 oocytes in the absence of extracellular Cl^- was 1723 ± 402 pmol X oocyte^{-1} X hour $^{-1}$. In oocytes microinjected with KCC1, $^{86}Rb^+$ uptake increased to 12,632 ± 2205 pmol X oocyte^{-1} X hour $^{-1}$, and the influx was Cl^- -dependent. The difference in the amount of uptake between KCC4 and KCC1 was statistically significant ($p < 0.05$). Although equal amounts of KCC4 and KCC1 cRNA were injected for all experiments, the relative expression level under hypotonic conditions was always greater for KCC4 than KCC1. In addition, for each KCC, the absolute uptake varied with each animal. Results of uptake experiments are presented as the percentage of Cl^--dependent $^{86}Rb^+$ uptake. Thus, 100% generally

represents the uptake observed in the KCC4 or KCC1 control group minus the uptake observed in the water-injected oocytes.

Uptake assays to detect K^+- Cl^- cotransport mediated by a hKCC2 were performed using similar methods. Figures 7A, 7B, and 8 depict functional expression of the hKCC2 transporter in both isotonic and hypotonic conditions.

Example 13

Kinetic Properties of KCC4 and KCC1

Figures 9A-9D summarize the kinetic characterization of KCC1, KCC3, and KCC4. The four KCCs differ in the sequence of key transmembrane segments (TMs), and were postulated to vary in affinity for K^+ and Cl^- (Mount et al. (1999) *J Biol Chem* 274(23):16355-16362). By plotting K^+-Cl^- cotransport as a function of the external concentration of both K^+ and Cl^- (shown for KCC3a in Figures 9A and 9B, respectively), the individual apparent affinity constants for these transported ions have been determined.

To determine and compare the kinetic properties of KCC4 and KCC1 in the same expression system, $^{86}Rb^+$ uptake was measured in KCC4- and KCC1-injected oocytes as a function of the concentration of each transported ion (Figure 9). Uptake assays were performed with K^+ or Cl^- fixed at 50 mM, and the concentration of the counterion was varied from 0 to 50 mM. Uptake assays were also performed in water-injected oocytes, and the mean values for water-injected oocytes were subtracted from corresponding KCC-injected oocytes in order to assess only the $^{86}Rb^+$ uptake mediated by each heterologously expressed isoform. As shown in Figure 6, $^{86}Rb^+$ uptake in water-injected oocytes was low, such that this correction was generally minor.

In the case of KCC4, $^{86}Rb^+$ influx increased concomitant with increased concentration of each transported ion. A plateau phase was reached at ion concentrations greater than 20–40 mM, compatible with Michaelis-Menten behavior. The calculated apparent K_m and V_{max} for extracellular K^+ concentration were 17.5 ± 2.7 mM and 32,370 ± 2,115 pmol x $oocyte^{-1}$ x $hour^{-1}$, respectively. The calculated apparent Km and Vmax values for extracellular Cl^- concentration were 16.12 ± 4.2 mM and 41,440 ± 4,174 pmol x $oocyte^{-1}$ x $hour^{-1}$, respectively. The Hill coefficient for both ions remained close to unity:

1.08 ± 0.2 and 1.06 ± 0.3 for K^+ and Cl^-, respectively. KCC1 also exhibited a similar Michaelis-Menten behavior. The apparent K_m and V_{max} in KCC1 were 25.5 ± 3.2 mM and 39,540 ± 2,199 pmol x oocyte $^{-1}$ x hour $^{-1}$ for extracellular K^+ and 17.2 ± 8.3 mM and 14,930 ± 2,822 pmol x oocyte $^{-1}$ x hour $^{-1}$ for Cl^-. Hill coefficients for K^+ (1.04 ± 0.13) and Cl^- (1.3 ± 0.5) in KCC1 also were close to unity.

Figure 10 presents line graphs depicting concentration dependence of K^+-Cl^- cotransport mediated by hKCC2, which was used to calculate ion affinities using analogous methods. Uptake assays were simultaneously assessed in water-injected oocytes. To determine $^{86}Rb^+$ uptake due to KCC2, the mean values for water groups were subtracted from the mean values for corresponding KCC2 groups. Uptake assays in hypotonic media were performed for 60 minutes with a fixed concentration of K^+ or Cl^- at 50 mM. The concentration of the counterion was varied from 0 to 50 mM as indicated. Lines were fit using the Michaelis-Menten equation, yielding a K_m for $^{86}Rb^+$ (substitute for K^+) and Cl^- of K_m 9.3 +/- 1.8 mM and 6.31 +/- 0.92, respectively. This cation K_m is similar to that reported for rat KCC2 analyzed in HEK293 cells (Payne et al. (1990) *Am J Physiol* 273:C1516-1525), however the chloride affinity is significantly higher (>50 mM reported for rKCC2). Of note, the measured chloride affinity for hKCC2 is closer to the chloride concentration of neurons (Hara et al. (1992) *Neurosci Lett* 143:135-138).

As shown in Figure 11, the slopes of activation of hKCC3a and hKCC3b by cell swelling, but that both isoforms begin to activate at an extracellular osmolality of 160 mOsm/kg. These differences in activation can be attributable to differences between predicted phosphorylation of their divergent amino termini (Mount et al. (1999) *J Biol Chem* 274:16355-16362).

As shown in Table 3, the four KCCs differ significantly in affinity for K^+ and Cl^-, due presumptively to variation within TM2 (cation affinity), and within TM4 and TM7 (anion affinity) (Mount (1999) *J Biol Chem* 274(23):16355-16362). Kinetic data for rat KCC2 are from Payne (1997) *Am J Physiol* 273:C1516-C1525, "xKCC" refers to the endogenous *Xenopus* oocyte transporter. Of note, as predicted by the sequence of TM2, the four KCCs fall

into two groups with respect to K^+ affinity, a low-affinity subgroup (KCC1 and KCC3) and a higher-affinity subgroup (KCC2 and KCC4).

Example 14

Anion Dependence of KCC1, KCC2, KCC3a, and KCC4

The anion selectivity of the three KCCs has also been determined, looking at the ability of anions other than Cl^- to support $K^+/^{86}Rb^+$ transport. These results indicate that the KCCs differ in this "anion series" of K^+-Cl^- cotransport. This data is summarized in Figures 12 and 13, as the percent activity (compared to chloride) for each anion. Again, this variability in anion series is likely due to variation with TM4 and TM7, segments thought to confer anion affinity on the cation-chloride cotransporters. See Mount et al. (1999) *J Biol Chem* 274(23):16355-16362; Isenring et al. (1998) *J Gen Physiol* 112(5):549-558.

It has been shown that some extracellular anions other than Cl^- can support ion translocation through the K^+-Cl^- cotransporter of both sheep and human erythrocytes (Payne et al. (1990) *Am J Physiol* 259:C819-827). Thus, $^{86}Rb^+$ transport by KCC4 and KCC1 was measure in the presence of different anions. The $^{86}Rb^+$ influx of KCC4- and KCC1-injected oocytes using an uptake solution containing 40 mM potassium gluconate and 10 mM KCl served as the reference activity for these experiments, as compared with uptake activity in oocytes exposed to medium containing 40 mM potassium gluconate and 10 mM of KBr, KH_2PO_4, KI, potassium gluconate, or KSCN. Figure 12 shows the percentage of KCC4 and KCC1 function when $^{86}Rb^+$ uptake assays were performed using these different anion substitutions. KCC4 shows high $^{86}Rb^+$ influx in the presence of 10 mM KCl. $^{86}Rb^+$ influx was still observed in the presence of other anions: 58 ± 9% with 10 mM KBr, 22 ± 5.9% with 10 mM KH_2PO_4, and 17 ± 3.8% with KI, whereas potassium gluconate and KSCN did not support transport. These results are in contrast to those observed in KCC1-injected oocytes, for which the order of anion-supported transport was Cl^- > SCN- = Br- > PO4-3 > I > gluconate.

Figure 13 shows the ion dependence of hKCC2 K^+-Cl^- cotransport determined by $^{86}Rb^+$ uptake assays performed in the presence of different

anion substitutions. KCC2 mediates K^+- Cl^- cotransport in a similar manner to KCC4 (see Table 3).

Example 15

Inhibitor Profile of KCCs

Figures 14-19 summarize the pharmacological characterization of the KCCs, examining the effect of a number of different inhibitors of anion and cation transport. A particularly important finding is the universal dependence of the anion inhibitors on the presence of extracellular K^+. Thus in Figures 14-19, results are expressed as percentage function (in comparison to swelling-induced activity) of the individual KCCs.

The effect of the loop diuretics furosemide and bumetanide was initially assessed using two different concentrations of extracellular K^+: 2 and 50 mM. In uptake medium with a K^+ concentration of 2 mM, relative KCC4 activity was 61 ± 3 and 90 ± 4% in the presence of 2 mM furosemide or bumetanide, respectively. Interestingly, the inhibition of KCC4 by loop diuretics was augmented when the uptake medium contained 50 mM K^+; under these conditions, the KCC4 activity was 9 ± 4 or 17 ± 4% in the presence of furosemide or bumetanide, respectively. In contrast, for KCC1 this effect of extracellular K^+ was not observed for furosemide and was marginal for bumetanide. KCC1 function in the presence of furosemide was 9 ± 2% in 2 mM K^+ and 18 ± 8% in 50 mM K^+ (p not significant), and in the presence of bumetanide it was 51 ± 12 versus 19 ± 7% in 2 and 50 mM K^+, respectively ($p = 0.05$; $t=1.99$). To further define the differences in the K^+ effect on the sensitivity to loop diuretics between KCC4 and KCC1, the inhibitory effect of furosemide and bumetanide was assessed at several concentrations of extra-cellular K^+ (Figure 14). The percentage inhibition of KCC4 by both furosemide and bumetanide was significantly affected by extracellular K^+ (Figure 14, upper panels). The minimal and maximal inhibition by both loop diuretics was observed at 2 and 6 mM, respectively; no further effect was observed at higher K^+ concentrations. In contrast, the percentage of KCC1 inhibition by either furosemide or bumetanide did not vary as a function of extracellular K^+ concentration (Figure 14, lower panels).

To define differences between the two KCCs in sensitivity to loop diuretics, 10 mM extracellular K^+ was used to assess the concentration curves for furosemide and bumetanide inhibition upon the Cl^--dependent $^{86}Rb^+$ uptake induced by KCC4 or KCC1. As Figure 15 illustrates, KCC4 exhibits apparent half-maximal inhibition (K = 0.5) values of ~900 μM for both furosemide and bumetanide. These are lower than the respective values for KCC1 (~180 μM for furosemide and bumetanide). Therefore, KCC4 clearly exhibits a lower affinity for loop diuretics than does KCC1. The inhibition of KCC1 by furosemide in Figure 14 suggests the possibility of a second affinity site for the loop diuretic. However, this inhibition fitted well to a Michaelis-Menten kinetics pattern with one inhibitor-binding site. The data did not fit to an equation with two binding sites.

Figure 16 is a line graph depicting dose-dependent inhibition of KCC2 by furosemide or bumetanide. Groups of oocytes microinjected with KCC2 cRNA were exposed to increasing concentrations of furosemide or bumetanide in the preincubation and uptake mediums, from 2 to 2000 μM. Uptake assays were performed in hypotonic conditions. Each point represents the mean ± standard error of at least 15 oocytes.

Figure 17 presents bar graphs depicting hKCC3b-mediated K^+-Cl^- cotransport in *Xenopus* oocytes, with minimal activity under isotonic conditions (220 mOsm/kg) and marked activation under hypotonic conditions (110 mOsm/kg). Open bars, uptake assays performed in a control medium containing 10 mM K^+ and 50 mM Cl^-; black bars, uptake assays performed in medium lacking Cl^-; gray bars, uptake assays performed in medium containing 2 mM furosemide.

The sensitivity of the KCCs to other inhibitors of red cell K^+-Cl^- cotransport was also assessed in oocytes injected with KCC4 or KCC1. Figure 18 illustrates the effect of 100 μM DIDS and 100 μM DIOA on the $^{86}Rb^+$ uptake induced by the microinjection of each KCC cRNA. The effect of extracellular K^+ concentration on the inhibition of cotransport was very dramatic for DIDS. When the concentration of extracellular K^+ was 2 mM, the addition of DIDS to the extracellular medium resulted in reduction of KCC4 function to 65 ± 10% (p

< 0.003) and of KCC1 to 85 ± 6% (p = 0.113, not significant). In contrast, when 50 mM of extracellular K^+ was used, DIDS resulted in significant decrease of KCC4 and KCC1 to 13 ± 4 and 13 ± 2%, respectively. The addition of 100 µM of DIOA to the extracellular medium also resulted in inhibition of the KCCs. However, inhibition of KCC4 was higher when extracellular K^+ was lower, although this was not the case for KCC1. DIOA is reportedly specific for K^+- Cl^- cotransport over Na^+-K^+-2Cl^- cotransport (Garay et al. (1988) *Mol Pharmacol* 33:696-701), and the same concentration of DIOA had no effect on the function of the Na^+-K^+-2Cl^- cotransport activity of *Xenopus* oocytes (Gamba et al. (1994) *J Biol Chem* 269: 17713-17722).

The effect of a 2 mM concentration of the thiazide diuretic trichlormethiazide on the percentage of chloride-dependent $^{86}Rb^+$ uptake was also measured. Surprisingly, given the supposed specificity of thiazides for Na^+-Cl^- cotransport (Rose et al. (1991) *Kidney Int* 39:336-352), KCC4 was moderately sensitive to trichlormethiazide. As observed for furosemide and DIDS, the higher the extracellular K^+, the higher the inhibition by thiazides, since in 2 mM of extracellular K^+ $^{86}Rb^+$ uptake was reduced to 79 ± 3%, and at 50 mM it was reduced to 57 ± 9%. This difference was significant (p < 0.01).

In KCC1-injected oocytes, trichlormethiazide reduced $^{86}Rb^+$ uptake by a statistically significant amount to 64 ± 4% in 2 mM K^+; this inhibitory effect was not statistically significant at 50 mM K^+ (74 ± 8% reduction in activity). Consistent sensitivity to trichlormethiazide is thus unique to KCC4. Independent studies have suggested that barium can inhibit renal K^+- Cl^- cotransporters (Greger & Schlatter (1983) *Pflugers Arch* 396:325-334; Amlal et al. (1994) *Am J Physiol Cell Physiol* 267:C1607-1625; Di Stefano et al. (1998) *Cell Physiol Biochem* 8:89-105). In view of such, the effect of 10 mM extracellular barium on the function of KCC4 and KCC1 was determined. Figure 19 shows that when 10 mM $BaCl_2$ was added to the uptake medium, KCC4-induced influx was reduced to 58 ± 4.3% of the uptake observed in KCC4-injected control oocytes. KCC1 function was only reduced to 79 ± 4.2%, hence the inhibitory effect of barium was significantly greater for KCC4 than for KCC1 (p = 0.01).

Example 16

Regulation of KCC4 and KCC1 by NEM

One of the most distinctive characteristics of K^+-Cl^- cotransport in several cells and species is activation by the alkylating agent NEM (Lauf et al. (1992) *Am J Physiol* 263:C917-932). The effect of NEM on $^{86}Rb^+$ influx in oocytes expression KCC4 or KCC1 was assayed under isotonic or hypotonic conditions. The uptake observed in KCC4- or KCC1-injected oocytes in isotonic medium was not different from the uptake in water-injected oocytes. However, the addition of 1 mM NEM in isotonic conditions resulted in a 5-fold activation of KCC4 (214 ± 12 pmol x oocyte $^{-1}$ x hour $^{-1}$ in the KCC4 control group versus 1062 ± 70 pmol x oocyte $^{-1}$ x hour $^{-1}$ in the NEM-treated group, ($p < 0.001$) and a 2.6-fold activation of KCC1 (120 ± 27 versus 319 ± 76 pmol x oocyte $^{-1}$ x hour^{-1} ($p < 0.05$) (Figures 20A and 20B). Of note, when uptake assays were performed in hypotonic medium, the addition of NEM resulted in a dramatic inhibition of both isoforms (Figures 20C and 20D), such that $^{86}Rb^+$ uptakes induced by KCC4 and KCC1 were reduced by 68% and 55%, respectively. In the same experiments, $^{86}Rb^+$ uptake due to the endogenous oocyte K^+-Cl^- cotransporter (H_2O-injected oocytes) was significantly increased when uptake assays were done under both isotonic and hypotonic conditions.

Example 17

Regulation of KCCs by Mercury

Mercury is known to inhibit several transporters via interaction with transmembrane or juxtamembrane cysteines (Kuwahara et al. (1997) *Biochemistry* 36:13973-13978; Jacoby (1999) *Am J Physiol* 277:C684-692). Given the inhibition of the KCCs by millimolar NEM at room temperature, presumed due to alkylation of cysteine residues (Mercado et al. (2000) *J Biol Chem* 275:30326-3034), the differential response of the KCCs to 50 μM mercury ($Hg\text{-}Cl_2$) was examined. KCC1 and KCC3a activated by hypotonic conditions are resistant to 50 μM mercury $Hg\text{-}Cl_2$, whereas KCC2 and in particular KCC4 are sensitive to this agent (Figure 21). An inspection of the amino acid sequence of mKCC4 reveals a total of five transmembrane cysteines that are not found in KCC1, KCC3, and/or KCC2. A mutant with four

of these five cysteines mutated (C256A/C469N/C565A/C647M) is still weakly sensitive to Hg-Cl_2 (quadruple mutant in Figure 22), whereas the corresponding quintuple mutant (C256A/C469N/C565A/C647M/C633S) is resistant (quintuple mutant in Figure 22). These results implicate cysteine-633, within transmembrane domain 12, in the sensitivity of mKCC4 to Hg-Cl_2. Of note, a cysteine residue in TM11 of NKCC1 is involved in the response of Na^+-K^+-$2Cl^-$ cotransport to mercury (Jacoby (1999) *Am J Physiol* 277:C684-692). This analysis supports the development of isoform-specific inhibitors of the KCCs, by targeting functionally important residues such as cysteine-633 of KCC4 and the equivalent residue in hKCC2, cysteine-613.

Example 18

Regulation of KCC Function by Phosphorylation

The red cell K^+-Cl^- cotransporters are thought to be phosphorylated by a volume-sensitive kinase under isotonic conditions, with dephosphorylation and activation by the serine-threonine phosphatases PP-1 and PP-2A (Bize et al. (1999) *Am J Physiol* 277:C926-936). The identity of this volume-sensitive kinase is unknown. However, insight could ultimately come from the assumption that this kinase or kinases also activates Na^+-K^+-$2Cl^-$ cotransport, a pathway that is stimulated by cell shrinkage and inhibited by staurosporine, NEM, and protein phosphatases (Lytle (1998) *Am J Physiol* 274:C1002-1010). Kelley et al. (2000) *J Membr Biol* 178:31-41 have investigated the role of myosin-light chain kinase (MLCK), another shrinkage-activated serine-threonine kinase, in the activation of K^+- Cl^- cotransport. They found modest activation by the MLCK inhibitor ML-7 of human red cell K^+-Cl^- cotransport at physiological osmolality. Since the kinetics of hKCC3 suggest that it is the dominant red cell K^+-Cl^- cotransporter, as disclosed herein, the effect of ML-7 on KCC3a expression in *Xenopus* oocytes was evaluated. Just at and below the threshold for activation of KCC3a (160-180 mOsm/kg), there is clear activation of K^+-Cl^- cotransport by 100 μM ML-7 (989 ± 115 pmol/oocyte/hour for KCC3a in the absence of ML-7 versus 1698 ± 298 in the presence of ML-7 at 160 mOsm/kg (Figure 23). There is also modest activation of hKCC3a by ML-7 at 180 mOsm/kg, however ML-7 has no effect on K^+-Cl^- cotransport under

isotonic conditions. Given this absence of an effect under isotonic conditions, it is likely that the kinase(s) inhibited by ML-7 are only part of the complex signaling cascade invoked by cell swelling. However, this approach illustrates the utility of the *Xenopus* expression system in studying the regulation of expressed KCCs.

The inhibition of protein phosphatases prevents the activation of red cell K^+-Cl^- cotransport by either cell swelling or NEM. Since the role of phosphatases in the control of the cloned KCCs is unclear, the effect of three inhibitors of protein phosphatases was evaluated. One assay included 100 nM calyculin A, which inhibits the function of protein phosphatases 1 and 2A. The relative role of specific phosphatases was assessed using okadaic acid at 1 nM, a concentration that only affects protein phosphatase 2A. Cypermethrin was used at 100 pM, a concentration in which this compound inhibits the function of protein phosphatase 2B. The addition of calyculin A completely prevents the activation of KCC4 and KCC1 by cell swelling (Figure 24). By contrast, neither okadaic acid, nor cypermethrin prevented this activation (Figure 24). These results indicate that protein phosphatase 1 is required for the activation of both KCC4 and KCC1 by cell swelling.

Figure 25A is a bar graph depicting the effect of shows the effect of the protein phosphatase inhibitor calyculin A (100 nM) on the isotonic K^+-Cl^- cotransport mediated by hKCC2. $^{86}Rb^+$ influx was assessed in a control group (white bars), in the absence of extracellular Cl^- (black bars), or in the presence 100 mM calyculin (hatched bars). Each bar represents the mean ± standard error of data obtained using at least 15 oocytes. Asterisk (*) indicates that $^{86}Rb^+$ influx was significantly reduced in the absence of Cl^-.

Figure 25B is a bar graph depicting the effect of shows the effect of the protein phosphatase inhibitor calyculin A (100 nM) on the swelling-induced K^+-Cl^- cotransport mediated by hKCC2. $^{86}Rb^+$ influx was assessed in a control group (white bars), in the absence of extracellular Cl^- (black bars), or in the presence 100 mM calyculin (hatched bars). Each bar represents the mean ± standard error of data obtained using at least 15 oocytes. Asterisk (*) indicates

that $^{86}Rb^+$ influx was significantly reduced in the absence of Cl^-, or in the combined absence of Cl^- and presence of calyculin.

Figure 26 is a bar graph depicting hKCC3b-mediated K^+- Cl^- cotransport in *Xenopus* oocytes microinjected with H_20 or KCC3b cRNA as indicated, in the presence of putative inhibitors. hKCC3b-mediated K^+-Cl^- cotransport is sensitive to furosemide treatment and blocked by Calyculin A treatment. Open bars, uptake assays performed in a control medium containing 10 mM K^+ and 50 mM Cl^-; hatched bars, uptake assays performed in medium lacking Cl^-; bars with horizontal stripes, uptake assays performed in the presence of 100 nM calyculin A; black bars, uptake assays performed in the presence of okadaic acid; gray bars, uptake assays performed in the presence of cypermetrin.

Example 19

Tissue Distribution of KCC3 and KCC4 Transcripts

RNA was extracted from mouse tissues (C57BL/6J strain) using guanidine isothiocyanate and cesium chloride. Total RNA (10 mg/lane) was size-fractionated by electrophoresis (5% formaldehyde, 1% agarose), transferred to a nylon membrane (Stratagene of La Jolla, California), and probed sequentially with ^{32}P-labeled randomly primed probes corresponding to full-length glyceraldehyde-3-phosphate dehydrogenase and nucleotides 4417B5062 of mKCC4 (3'-UTR). Human multiple-tissue Northern blots containing 2 mg/lane poly(A)+ RNA (Clontech of Palo Alto, California) were hybridized to probes generated by PCR from the 3'-UTRs of hKCC4 (nucleotides 4598-4957) and hKCC3 (nucleotides 3624-4185) and to a human beta-actin probe. Hybridization for all blots was performed overnight at 42°C in 4X SSCP, 40% formamide, 4X Denhardt's solution, 0.5% SDS, and 200 mg of salmon sperm DNA, and membranes were washed twice for 10 minutes at room temperature in 2x SSCP, 0.1% SDS and twice for 1 hour at 65°C in 0.1X SSCP, 0.1% SDS. Exposure times varied.

Northern blot analysis was performed with probes derived from the 3'-UTRs of KCC3 and KCC4. KCC4 probes detect 5.3-kb transcripts in a number of tissues, most prominently in the heart and kidney. Very little KCC4 transcript is detectable in adult brain. KCC3 has a more restricted expression pattern,

with significant amounts of transcript found only in muscle, brain, lung, heart, and kidney. At least two different transcripts of 6B7 kb hybridize to KCC3 probes, consistent with alternative splicing.

Human KCC2 is heavily induced during the *in vitro* differentiation of human NT2 teratocarcinoma cells, which do not express this transporter in the undifferentiated state, into "NT2-N" neuronal cells (Pleasure & Lee (1993) *J Neurosci Res* 35(6):585-602) (Figure 27A). NT2-N cells are an increasingly well-characterized neuronal cell model, and are known to express several subtypes of $GABA_A$ receptors (Neelands et al. (1998) *J Neurosci* 18(13):4993-5007; Neelands et al. (1999) *J Neurosci* 19(16):7057-7065) as well as multiple neurotrophin receptors (Piontek et al. (1999) *J Neurochem* 73(1):139-146). As such these cells will provide an important cell model for understanding the function, post-transcriptional regulation, and transcriptional regulation of human KCC2.

A Northern blot of mouse tissues probed with both a KCC3 exon 1a-specific and a KCC3b exon 1b-specific probe is shown in Figure 27B. KCC3a is particularly abundant in brain and muscle, whereas KCC3b is most abundant in kidney, indicating that the two promoters are differentially regulated. RT-PCR of mouse tissues with primer in exons 1a and 4 is shown in Figure 27C. Two bands are amplified, corresponding to alternative splicing of exon 2; the shorter band corresponds to isoforms lacking this 45 base-pair cassette exon. Sequence data for mouse and human KCC3a lacking exon 2 (KCC3a-2m) is included in SEQ ID NOs:3-6.

Example 20

Generation of KCC3 and KCC4 Antibodies

Polyclonal antibodies were produced by subcutaneous injection of a KLH-coupled KCC3-specific peptide (KKARNAYLNNSNYEEGDEY; SEQ ID NO:116) to two New Zealand White rabbits (Quality Control Biochemicals of Hopkinkon, Massachusetts). This antigenic peptide is located within the putative intracellular amino-terminal tail of the cotransporter and shows no homology with the corresponding regions of the other three K^+-Cl^- cotransporter isoforms, KCC1, KCC2, and KCC4, with the two Na^+-K^+-$2Cl^-$ cotransporters,

NKCC1 and NKCC2, or with the Na^+-Cl^- cotransporter, NCC. Moreover, BLAST alignments against the non-redundant and EST databases do not detect significant homology with unrelated proteins. Finally, the peptide is not a predicted substrate for major protein kinases and was predicted to be highly antigenic using MACVECTOR™ software (available from Oxford Molecular, Inc. of Campbell, California). Antiserum from one rabbit with high titer was affinity purified by linking the peptide to AFFI-GEL™ 15 support (BioRad of Hercules, California) and by incubating the immune serum for two days at 4°C with the Affi-gel-peptide complex eluted in 5xphosphate-buffered saline (PBS). After washing in 5xPBS, specific antibodies were eluted with 0.1 M sodium citrate (pH 2.5), neutralized with 1 M Tris (pH 8.8) and dialysed in 1X PBS overnight at 4°C. The purified antibody was concentrated using a 30-kDa cut-off CENTRIPULUS™ column (Amicon of Beverly, Massachusetts) and aliquots were stored at -20°C.

Western blots of mouse membrane protein using anti-KCC3 immune serum detected a broad band representing a protein around 160-170 kDa, which was not observed in preimmune serum from the same rabbit (Figure 28, lanes 1 and 2). When the immune serum was purified using specific antigen, only the 150-170 kDa band was detected (Figure 28, lane 3). Rapid exposure of the Western blot revealed that the broad signal is composed of two distinct bands (Figure 28, lane 2). The strong signal observed when using the affinity-purified antibody was not observed when the antibody was preabsorbed with specific antigen (Figure 28, lane 4), demonstrating the specificity of the ~160 kDa signal as KCC3 protein.

Example 21

Tissue Distribution of KCC3 and KCC4 Proteins

Western blots using the amino-terminal KCC3-specific antibody, generated to a peptide antigen from exon 3 (KKARNAYLNNSNYEEGDEY; SEQ ID NO:116), are shown in Figures 27D and 27E. Although reactivity has not been tested against the five KCCs heterologously expressed in *Xenopus* oocytes, the peptide antigen is not found in the KCC1, KCC2 or KCC4 sequences, and does not detect other proteins in stringent BLAST searches

(Altschul et al. (1997) *Nucleic Acids Res* 25(17):3389-3402) of the non-redundant and EST databases. The KCC3 antibody reacts with proteins of ~160 kDa in several tissues, including brain and kidney (shown in Figure 27D, for renal cortex and outer medulla (OM), as well as brain). This reactivity is abolished when antibody is pre-incubated with peptide antigen ("IAB" sample, shown for renal cortex in Figure 27D). KCC3 protein is also detected in membrane samples from murine red cells (RBC lane in Figure 27E), and from a mouse proximal tubule cell line (Loghman-Adham et al. (1997) *Kidney Int* 52(1):229-239) (tsMPT lane in Figure 27E) and human umbilical vein endothelial cells (HUVEC lane in Figure 27E). The presence of KCC3 in red cells suggests that KCC3 contributes to red cell K^+-Cl^- cotransport activity (Lauf et al. (1992) *Am J Physiol* 263(5 Pt 1):C917-932).

An amino-terminal KCC4-specific antibody, generated to a peptide antigen from exon 1 (AERTEEPESPESVDQTSP; SEQ ID NO:117), detects a broad band of proteins between 160 and 180 kDa in molecular mass (Figure 27F). In renal cortex and outer medulla (OM), two separate bands can be resolved, with the lower mass band predominant in cortex and the higher mass band predominant in outer medulla (Figure 27G). This heterogeneity can be due to differential glycosylation, but is more likely generated by as-yet-uncharacterized alternative splicing. Finally, as in the case of the KCC3 antibody, reactivity is abolished by immunoabsorption with peptide antigen (Figure 27H).

Figures 27A-27H show that the four KCCs have distinct but overlapping tissue distributions. KCC1 and KCC4 are widely expressed (Gillen et al. (1996) *J Biol Chem* 271(27):16237-16244; Mount et al. (1999) *J Biol Chem* 274(23):16355-16362), KCC3 has a slightly more restricted expression pattern (Mount et al. (1999) *J Biol Chem* 274(23):16355-16362), and KCC2 is restricted to neurons in the central nervous system and retina. See Lu et al. (1999) *J Neurobiology* 39:558-568; Williams et al. (1999) *J Biol Chem* 274(18):12656-12664.

Expression of KCC3 protein in mouse brain is particularly prominent in the cell bodies and processes of oligodendrocytes, and is found in white matter

tracts throughout the CNS, including the corpus callosum. This expression pattern further strengthens the genetic association with Andermann's syndrome (Casaubon et al. (1996) *Am J Hum Genet* 58(1):28-34) and familial spastic paraparesis (Martinez Murillo et al. (1999) *Neurology* 53(1):50-56). KCC3 is also expressed at the basolateral membrane of choroid plexus cells, however evidence for neuronal expression is still equivocal. Human KCC3 is however induced during the one-month *in vitro* differentiation of NT2 teratocarcinoma cells into NT2-N neuronal cells. NT2-N cells express hKCC2 (Figure 27A). Human neurons thus have at least the potential to express both KCC2 and KCC3. KCC4 is expressed at very low levels in brain, however immunolocalization indicates focal expression in CA1 neurons within the hippocampus.

Example 22

Expression of KCC3 and KCC4 in Kidney

Immunofluorescence of mouse and rat kidney with the KCC3 antibody reveals expression at the basolateral membrane of proximal tubule, from S1 to S3; immunoabsorbed controls are negative. Co-immunofluorescence with distal nephron markers (antibodies to NKCC2, NCC, H-ATPase, *etc.*) indicates that KCC3 does not localize to the distal nephron. Despite expression in HUVEC cells, KCC3 is not detected in renal arteries. Like KCC3, KCC4 is heavily expressed at the basolateral membrane of proximal tubule cells, however expression is heaviest within S1 and weakest in S3. Neither KCC3 nor KCC4 are expressed in the glomerulus, whereas KCC1 is reportedly expressed in mesangial cells (Liapis et al. (1998) *Am J Physiol* 275(6 Pt 1):C1432-1437).

Unlike KCC3, KCC4 is expressed in the distal nephron, and co-immunofluorescence indicates expression at the basolateral membrane of the entire thick ascending limb (TAL), as well as in macula densa and distal convoluted tubule (DCT). Co-localization with aquaporin-1 suggests that descending thin limbs, thought to contain swelling-activated K^+-Cl^- transport (Lopes et al. (1988) *Proc Natl Acad Sci USA* 85(8):2873-2877), do not express KCC4 (Figures 29I-29J). Finally, co-localization with H^+-ATPase indicates

expression at the basolateral membrane of type A intercalated cells, cells that play a pivotal role in renal acid secretion.

The intra-renal localization of KCC3 and KCC4 settles the longstanding controversy regarding the presence or absence of K^+-Cl^- transport at the basolateral membrane of proximal tubule (Seki et al. (1993) *J Clin Invest* 92(3):1229-1235) and thick ascending limb (Di Stefano et al. (1998) *Cell Physiol Biochem* 8(1-2):89-105; Hurst et al. (1992) *Am J Physiol* 263(2 Pt 2):F262-267). The expression of KCC4 at the basolateral membrane of type A intercalated cells indicates an unexpected role in distal acid secretion.

Example 23

Expression of KCC3 Isoforms in Brain

Isoform-specific cDNA probes were used to examine the expression of KCC3a and KCC3b. The two isoforms are also distinguished by size, the KCC3a transcript being slightly larger than the KCC3b transcript (Figure 29A). KCC3a is abundantly expressed in brain, whereas KCC3b is predominantly expressed in kidney. Total RNA was further isolated from specific brain regions or structures, including choroid plexus, hypothalamus, cerebellum, brainstem, cerebral cortex, and white matter. KCC3a was detected at equivalent levels in all noted brain regions, with the exception of reduced expression in the choroid plexus (Figure 29B).

Brain and kidney from C5b6 mice were dissected and fresh tissue was frozen in liquid nitrogen. Total RNA was extracted using the guanidine isothiocyanate method, as described previously (Delpire et al. (1994) *J Biol Chem* 269:25,677-25,683). Poly(A)+ RNA was purified from total RNA using RNeasy silica gel columns (Qiagen of Valencia, California). Total RNA (10 μg/lane) or poly(A)+ RNA (3 μg/lane) was separated on 1% agarose-0.63% formaldehyde gel, transferred onto nylon membrane, and probed with mouse-specific ^{32}P-labeled probes consisting of 1a- or 1b-specific DNA sequences. Full-length cDNAs have been cloned from mouse KCC3a and KCC3b (GenBank Accession Nos. AF21185 and ZF211855, respectively). After overnight hybridization at 42°C in a formamide-containing hybridization solution,

the membranes were washed at high stringency (65°C) and exposed to autoradiography.

To determine the relative abundance and distribution of KCC3 proteins in brain, microsomal protein from the cerebral cortex, hippocampus, diencephalons, brainstem, and cerebellum were used to prepare a Western blot, and the blot was analyzed using anti-KCC3 purified antibody. KCC3 protein is abundant in all regions of the brain examined (Figure 30A). KCC3 signal was lower in the hippocampus in two of three Western blot analyses. Dissection of the white matter tracts of the internal and external capsules and corpus callosum indicated that KCC3 is highly expressing in these myelinated pathways (Figure 30B).

For preparation of the Western blot, brain, spinal cord, dorsal root ganglia and peripheral nerves from C47b6 mice and whole brain from rats of varying age were dissected and homogenized in sucrose buffer (0.32 M sucrose, 5 mM Tris-HCl, pH 7.5, 2 mM EDTA) with a Teflon pestle. Microsomal protein was obtained by successive centrifugation at 3000 g, 20,000 g and 100,000 g. The high-speed pellet was resuspended in a buffer containing 5 nM Tris-HCl (pH 7.5) and 2 mM EDTA. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difluoride membranes (BioRad of Hercules, California). Except when indicated, membranes were probed with affinity-purified KCC3 antibody at a dilution of 1:1000. Proteins were detected using enhanced chemiluminescence (Amersham of Arlington Heights, Illinois).

To detect KCC3 protein in tissues, brain, spinal cord and peripheral nerves from C57b6 mice were dissected and fixed overnight in 4% paraformaldehyde in PBS at 4°C. Tissues were then washed with PBS and cryoprotected with 30% sucrose in PBS. For indirect immuofluorescence, 7-10 μm frozen sections were thaw mounted on SUPERFROST PLUS™ slides (VWR Scientific of West Chester, Pennsylvania). Sections were then treated with 1% SDS and 8% 2-mercaptoethanol for 5 minutes, washed in PBS, blocked for 30 minutes at room temperature with PBS, 1% bovine serum albumin (BSA), followed by incubation with KCC3 affinity-purified antibody or

antibody preabsorbed with the antigenic peptide, all at dilutions of 1:200 in PBS-1% BSA, overnight at 4°C. After washes in PBS, slides were incubated with Cy3-conjugated goat anti-rabbit immunoglobulin G (Jackson Immunoresearch of West Grove, Pennsylvania) diluted 1:1000 in PBS, 1% BSA for 1 hour at room temperature in the dark. They were subsequently washed with PBS and mounted with Vectashield (Vector Labs of Burlingame, California). For double-labeling experiments, sections were incubated successively with anti-KCC3 antibody overnight followed by Cy3-conjugated secondary antibody and then with monoclonal anti-microtubule-associated protein 2 (anti-MAP2) antibody or anti-MBP antibody overnight, followed by fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (Jackson Immunoresearch of West Grove, Pennsylvania). Sections were analysed with a Nikon Eclipse E800 microscope equipped with an Optronics DEI-750 color CCD camera (Optronics Engineering of Goleta, California) coupled to an IBM-compatible 200 MHZ computer, connected to a color Tektronix Phaser 450 printer (Tektronics of Wilsonwill, Oregon).

For antigen retrieval, frozen brain sections were thaw mounted on SUPERFROST PLUS™ slides, air-dried at room temperature, treated with 100% ethanol for 5 minutes at room temperature, air-dried at 42°C and treated with 20 g/ml proteinase K in PBS, 1% BSA for 4 minutes at 37°C. After two washes in PBS, sections were treated for 5 minutes with 1% SDS and 8% 2-mercaptoethanol, and processed as indicated above.

KCC3 expression was also examined in other nervous system regions, including spinal cord, dorsal root ganglia, and peripheral nerves. Microsomal protein was prepared form the noted region. Western blot analysis revealed robust expression of KCC3 in the spinal cord (Figure 30B). By contrast, low levels of KCC3 were detected in dorsal root ganglia, and only trace amounts were detected in sciatic and trigeminal nerves (Figure 30B).

To examine the postnatal developmental profile of KCC3 expression, protein derived from brains isolated at postnatal day 1 (P1), P7, P14, P21, and adult (>P60) was analyzed by Western blot analysis (Figure 30C). KCC3 levels are low at birth and increase significantly during postnatal maturation. KCC3

levels in the adult brain are 30-fold higher than in P1 brain. At each age, double protein bands of 155 and 165 kDa were detected, confirming the specificity of the antibody throughout postnatal development. The presence of KCC3 in structures rich in white matter, together with elevated levels during postnatal development, implicate KCC3 in myelination and related biological activities.

To determine the anatomical distribution and cellular localization of KCC3 in the brain and spinal cord, polyclonal anti-KCC3 antibody was used to label 7-10 μm sections of adult mouse and spinal cord (Figure 31). In the spinal cord, KCC3 expression was highest in highly myelinated tracts of the dorsal columns. This pattern was similar to the distribution of oligodendrocyte markers CNPase and myelin basic protein. Double-labeling using anti-KCC3 and anti-CNPase antibodies revealed substantial co-staining. White matter tracts of the ventrolateral columns were similarly labeled with both KCC3 and CNPase. KCC3 immunostaining was absent when the antibody was preabsorbed with specific antigen.

Immunolocalization of KCC3 in the brain was initially impaired by the seeming inaccessibility of the target epitope, probably due to protein-protein interactions. Using a standard immunostaining protocol, a specific signal was detected only at the base of the choroid plexus epithelium. This staining was prevented by preincubation the antibody with antigenic peptide. A high uniform background signal was also observed.

To uncover the KCC3 epitope in brain, tissue sections were treated with proteinase K (Hardt et al. (2000) *J Comp Pathol* 122:43-53). In the forebrain, high levels of KCC3 were observed in white matter tracts (*e.g.*, the corpus callosum, Figure 32A). A predominant proportion of KCC3 in the corpus callosum co-localized with the oligodendrocyte markers CNPase (Figure 32B) and myelin basic protein, although a number of KCC3-positive cell bodies were devoid of CNPase reactivity (Figure 32B, arrows). KCC3 was also detected in packed cell layers of the hippocampus (CA1, Figures 32A, 32D) and cortex (Figures 32E, 32F), suggesting neuronal expression. Neuronal staining was distinguished by : (i) a high density of cell bodies labeled in the CA1 layer of the

hippocampus (Figure 32A, 32C) and dentate gyrus (Figure 32D); and (ii) the presence of labeled cells in the cerebral cortex, including a few cells in molecular layer I, a greater number of cells in layer II, and dispersed cells in deeper layer (Figure 32F). In deeper cortical layers, KCC3 was detected in pyramidal neurons, which can be identified by their distinctive morphology (Figure 32E).

In the cerebellum, KCC3 was detected in Purkinje neurons and their axons, whereas KCC3 was not detected in granular neurons. White matter tracts were also labeled with anti-KCC3 antibody. The levels of KCC3 in white matter were relatively high in vertical tracts located in the brainstem. KCC3 in white matter co-localizes with the oligodendrocyte marker CNPase, demonstrating the association of KCC3 with myelin sheaths.

Large, MAP2-positive cells in the brainstem also showed KCC3 immunoreactivity.

Example 24

KCC2 Knockout Mice

A mouse strain genetically deficient in KCC2 has been generated, and homozygous mice were shown to suffer from early neonatal mortality. The targeting strategy for this mouse strain is shown in Figure 33A. This figure shows the structure of the 5' end of Slc12a5/KCC2 gene, position of the 5' probe and structure of the DNA fragment inserted into the gene. The construct was created using pPNT, a vector containing both neomycin and thymidine kinase genes under phosphoglycerate kinase-1 (PGK-1) promoter. A 2.1-kb Sph I-Xho I fragment (left arm) was ligated upstream of the neomycin cassette, and a 7-kb BamH I fragment (right arm) was inserted between neomycin and thymidine kinase. Figure 33B shows Southern-blot analysis of embryonic stem (ES) cell genomic DNA digested with Nhe I and BamH I; the 3-kb band represents the control gene and the 4.5-kb band originates from the mutant gene.

As shown in Figure 33C, PCR analysis of genomic DNA confirms the presence of the mutant gene in two ES cell mutants (1F12 & 2B9). Position of

the PCR product is indicated (arrowhead). The upper band represents the primers.

Figure 33D charts the life expectancy of wild type mice, heterozygote $KCC2^{+/-}$ mice, and homozygous $KCC2^{-/-}$ mice. Following birth, pups were examined and counted twice a day. Dead pups were removed and genotyped by PCR. The striking death rate of homozygote pups between day 11 and 17 is shown, and all homozygote mutants died by day 17.

Figure 33E shows photographs of seizing $KCC2^{-/-}$ mice. Homozygote mutant and control mice were placed upside-down. While the control mouse (bottom right) turned immediately back right side up, the homozygote mutants started to seize. Note the general stiffness of the limbs of $KCC2^{-/-}$ mutant animals.

To investigate the neural defects responsible for seizure activity in KCC2 mutant animals, the brains of epileptic $KCC2^{-/-}$ mice was evaluated histologically. When compared to the brains of control animals, $KCC2^{-/-}$ brains display signs of injury, including increased immediate early gene expression (*e.g., fos*) and a loss of interneuronal populations (*e.g.*, parvalbum-positive interneurons).

A reduced threshold for induction of seizure activity was also observed in $KCC2^{+/-}$ heterozygous mice. Seizures were induced in wild type and litter-matched $KCC2^{+/-}$ mice by daily injections of 60 mg/kg pentylenetetrazole (PTZ), a blocker of the $GABA_A$ receptor. Animal behavior was observed during a one-hour interval following PTZ injection, and the severity of seizure induction was scored according to the following indicators: score = 0, absence of seizure acitivity; score = 1, ear and facial twitching; score = 2, myoclonic body jerks; socre = 3, clonic forelimb convulsions with tonic extension episode and status epilepticus; score = 5, death. Mice assigned a score of 3, 4, or 5 were removed from the group. Figure 34 summarizes the number of mice assigned a socre of 1 or 2 as a function of time. A significant difference between wild type and $KCC2^{+/-}$ heterozygous mice was observed, corresponding to an approximately 2-fold increase in the susceptibility of $KCC2^{+/-}$ heterozygous mice to seizure activity.

Example 25

KCC3 Knockout Mice

Figure 35 shows the targeting construct for the generation of a KCC3 knockout mouse strain. A PCR strategy was used to remove exon 3 and to introduce a Xho I site within the 1450 base pair Sph I fragment. The IRES/bgeo fragment was inserted in the KCC3 gene at the Xho I site. This mutation deletes exon 3, which is not alternatively spliced and is utilized by both KCC3a and KCC3b transcripts.

$KCC3^{-/-}$ mutant mice display several abnormalities, including uncoordinated gait/locomotion, a nervous sytem defect manifest as reduced exploratory behavior, prepulse inhibition, and reduced myelination of peripheral axons. Human KCC3 maps to human chromosome 15q15, and this region is genetically linked with periodic catatonia, a subtype of schizophrenia (Stober et al. (2000) *Am J Hum Genet* 67:1201-1207). The phenotypes observed in $KCC3^{-/-}$ mice support the involvement of KCC3 in the genesis of schizophrenia. The early locomoter phenotype and the demyelination of peripheral nervies is similar to that observed in patients afflicted with ACCPN (peripheral neuropathy with or without agenesis of the corpus callosum) (Casaubon et al. (1996) *Am J Hum Genet* 58:28-34). Further, the strong prepulse inhibition phenotype demonstrated by $KCC3^{-/-}$ and $KCC3^{+/-}$ mice is suggestive of the schizophenia-like psychotic symptoms that have been described for ACCPN patients (Casaubon et al. (1996) *Am J Hum Genet* 58:28-34). The genomic structure of human KCC3, disclosed herein, will facilitate diagnosis of patients with schizophrenic disorders.

$KCC3^{-/-}$ mice develop an abnormal posture and gait prior to weaning (postnatal day 21). The limbs of $KCC^{-/-}$ animals are weak, manifest as a low posture and uncoordinated limb movements. To further describe this sensorimotor defect, three behavioral tasks that focus of locomotor performance (the rotorod task, the wire hang task, and the beam task) were employed, each described briefly herein below. Collectively, these data indicate locomotor deficits, involving both strength and coordination, in $KCC3^{-/-}$ mice but not in $KCC3^{+/-}$ heterozygous mice.

The rotorod task involves placing an animal and scoring the animal's latency to fall. Rotorod trials were conducted 3 times per day for three days, and this test series was performed when animals were 1 month and 4 months of age. Figure 36A summarizes the performance of the test animals on the third day of each test series. No significant difference between wild type and $KCC3^{+/-}$ heterozygous mice was observed. By contrast, $KCC3^{-/-}$ mutant mice failed the rotorod test as they fell shortly after being placed on the wheel.

The wire hang task involves presenting a wire to the animal's forelimbs. A normal response is characterized by gripping of the wire and pulling the body up so that the hindlimbs and tail also engage the wire. As shown in Figure 36B, wild type and $KCC3^{+/-}$ heterozygous mice grabbed the wire without difficulty, whereas $KCC3^{-/-}$ mice performed poorly. Results were consistent when animals were tested at 1 month and 4 months of age.

The beam task involved placing the mouse on a narrow beam and measuring the latency passed until the animal fell from the beam. As shown in Figure 36C, wild type and $KCC3^{+/-}$ heterozygous mice maintained position on the beam for one minute (the duration of the test), whereas $KCC3^{-/-}$ mice fell rapidly. Results were consistent when animals were tested at 1 month and 4 months of age.

To assess neural contributions to the locomotor phenotypes in $KCC3^{-/-}$ mutant mice, an activity chamber assay was performed. Wild type, $KCC3^{+/-}$ heterozygous mice, or $KCC3^{-/-}$ mutant mice were individually placed in an activity chamber, and the distance traveled and/or the number of rearing occurrences by the mouse was recorded in 4 contiguous 5-minute periods. The results are summarized in Figures 37A and 37B. Wild type mice show a high level of activity durinig the initial 5-minute period and a progressively decreased level of activity during each subsequent period. This behavior constitutes a normal pattern of exploration of a new environment followed by habituation. $KCC3^{-/-}$ mutant mice fail to explore their environment and also fail to habituate, as evidenced by a submaximal and constant level of activity. $KCC3^{+/-}$ heterozygous mice displayed reduced, although not absent, exploration and habituation activity. Thus, in $KCC3^{+/-}$ heterozygous animals, reduced

exploratory (Figure 37A) and rearing (Figure 37B) behavior is observed in the absence of measurable locomoter defects (Figures 36A-36C), suggesting that their compromised performance in the activity chamber assay is unrelated to locomoter performance.

KCC3$^{-/-}$ mice were also evaluated using a prepulse inhibition test to detect schizophrenia-like behavior. The test is based on the ability of a prepulse (in this case, an acoustic prepulse) to inhibit a sensory pulse. A wild type animal will learn to anticipate the sensory pulse based on the prepulse, wherease a schizophrenic animal will fail to learn the association. Startle response to an acoustic stimulus is recorded, and a prepulse of lower intensity is presented 100 milli-seconds prior to the acoustic pulse. The results of these experiments are summarized in Figure 38. No differences in startle response were observed between wild type and KCC3$^{-/-}$ mutant mice, indicating that auditory functions are not disrupted in KCC3$^{-/-}$ mutant mice.

The human KCC3 gene shows genetic linakge with ACCPN, a disorder characterized in part by demyelination of peripheral nerves. To assess myelination in KCC3$^{-/-}$ mice, thick (1 μm) sections of sciatic nerve derived from wild type and KCC3$^{-/-}$ mutant mice were prepared for hisotpathological examination. Large axons isolated from wild type mice show thick myelination (Figures 39A-39B). By contrast, thinner myelin sheaths, degenerating axons, and numerous axons with a ring-like appearnce were observed in axons derived from KCC3$^{-/-}$ mutant mice (Figures 39C-39D).

Example 26

Identification of a KCC2 Polymorphism

Whereas targeted deletion of the mouse KCC2 gene results in repetitive seizures and early neonatal lethality (Delpire & Lovinger (2000) *J Neurosci* 26:1148), the region of chromosome 20q13 containing the hKCC2 gene is not linked to hereditary epilepsy syndromes. However, it remains possible that variability in the expression and function of hKCC2 plays a role in human disease. Of note, the NRSE observed in mouse KCC2 is conserved in the human gene, just 5′ of a complex $(CA/GT)_n$ repeat (Figure 40). To determine whether this repeat is polymorphic, primers flanking this region (SEQ ID

NOs:114-115) were used to amplify genomic DNA from several individuals. Amplification conditions consisted of a 5-minute denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a final 5-minute extension at 72°C. The sequence of both alleles from several individuals is shown in Figure 41, and reveals that the repeat is in fact polymorphic. This repeat might affect transcriptional regulation of the hKCC2 gene by affecting the binding of NRSF. Similar dinucleotide repeats within or near the regulatory elements of other genes have been shown to have polymorphic effects on transcription (Gebhardt et al. (1999) *J Biol Chem* 274:13176-13180; Shimajiri et al. (1999) *FEBS Lett* 455:70-74). Such genetic variability in transcription of the hKCC2 gene can impact on human seizure disorders and/or their treatment, or on other disorders that affect neuronal chloride homeostasis and the response to GABA (van den Pol et al. (1996) *J Neurosci* 16:4283-4292). Human NT2-N neuronal cells, which we have shown to express hKCC2, will be an invaluable resource in the transcriptional characterization of hKCC2, since this is evidently the only cell line that expresses KCC2 (Williams et al. (1999) *J Biol Chem* 274:12656-12664).

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The publications and other materials listed below and/or set forth by author and date in the text above to illuminate the background of the invention, and in particular cases, to provide additional details respecting the practice, are incorporated herein by reference. Materials used herein include but are not limited to the following listed references.

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It will be understood that various details of the invention can be changed without departing from the scope of the invention. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation--the invention being defined by the claims.